AD_____

Award Number: W81XWH-06-1-0413

TITLE: Identification of Genes Regulating the Development of Breast Cancer

PRINCIPAL INVESTIGATOR: Hua Wang, Ph.D.

CONTRACTING ORGANIZATION: University of Wisconsin-Madison
Madison, WI, 53705

REPORT DATE: April 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
14-04-2008	Annual	15 MAR 2007 - 14 MAR 2008

4. TITLE AND SUBTITLE
Identification of Genes Regulating the Development of Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0413

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Hua Wang, Ph.D.

Email: hwang@humonc.wisc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Wisconsin-Madison
Madison, WI, 53705

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We have identified four different modifiers of Apc_{min} that affect mammary tumor number or mammary tumor latency (Mmom1-Mmom4). To further investigate the molecular mechanisms of mammary modifiers, Mmom1 and Mmmom2 congenic mice were produced and tested for the effect on mammary tumor number or mammary tumor latency. The effect of Mmom2 on mammary tumor latency was confirmed in the congenic mice and the region containing Mmom2 was narrowed into a 10cM region. We also show in this report that genetic background can determine the mammary tumor types in Apc_{min} mice and we propose a model to explain how mammary modifiers could affect mammary tumor development.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	33	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

Introduction

Breast cancer develops through multiple steps which are rigorously controlled by genetic factors. It is essential to identify and characterize genes controlling the development of breast cancer to better understand factors affecting tumor susceptibility and contribute to better diagnosis and treatment. We are using a well characterized mouse model, *Apc* $^{Min/+}$ mice, to identify genes important for breast cancer progression and development. *Apc*$^{Min/+}$ mice are predisposed to both intestinal and mammary tumors due to a mutation on the tumor suppressor gene *Apc*. Genetic background affects the susceptibility to mammary tumor development in *Apc*$^{Min/+}$ mice. Upon exposure to the alkylating agent ENU (Ethylnitrosourea), more than 90% of B6-*Apc*$^{Min/+}$ female mice develop mammary squamous cell carcinomas, with an average 3.3 tumors per mouse and a mean latency of about 56 days. FVBB6 F1 *Apc*$^{Min/+}$ mice are resistant to mammary tumors but susceptible to focal alveolar hyperplasias under the same treatment . This suggests that FVB carries alleles at modifier loci of *Apc*^{Min} that act dominantly to affect the progression of hyperplasias to tumors. In this grant, we aim to use genetic analysis to identify mammary modifiers.

Body

A: Generate and test the mammary modifier congenic mice.

Four mammary modifiers have been identified to affect mammary tumor number or tumor latency. As a result for this funding, a paper was published on *Cancer Research* on Dec 2007 (See attachments).

To further study the molecular mechanisms of mammary modifiers, congenic mice were produced to contain modifiers on chromosome 9 (*Mmom1*) or 4 (*Mmom2*). Preliminary results from N6 *Mmom1* congenic mice did not show significant effect on either tumor number or tumor latency (Data not shown). However analysis of N10 *Mmom2* congenic mice confirmed the effect on mammary tumor latency and narrowed *Mmom2* into a 10cM region.

We generated *Mmom2* congenic mice for the region from *D4Mit193* (7.5 *cM*) to *D4Mit13* (71 *cM*) from FVB on the B6 background. At the N8-N9 generations, we selected *Mmom2* congenic female breeders which were heterozygous around the marker *D4Mit82* to cross with B6 $Apc^{Min/+}$ male mice. The results presented here are from a total 146 N9-N11 *Mmom2* congenic mice.

D4Mit82 was the peak marker on chromosome 4 identified in our backcross analysis and was significantly associated with mammary tumor latency and suggestively associated with tumor multiplicity. We first tested to see if any single marker was associated with tumor latency or tumor number in the congenic mice (Table 1). In this single marker analysis, *D4Mit82* has a significant affect on tumor latency (P=0.0057) but not tumor number (P=0.34) when comparing *BF* heterozygous with *BB* homozygous mice. Another marker, *D4SNP483*, is also associated with tumor latency (P=0.0049) but not tumor number (P=0.61) in the same analysis. The effect of *D4Mit82* and *D4SNP483* on tumor latency is illustrated in Figure 1A and 1B. These results support the existence of *Mmom2* on chromosome 4.

We identified several groups of congenic mice carrying different regions of the congenic interval to define the minimal region containing *Mmom2*. The *Mmom2-A* group mice carry B6 alleles from *D4Mit193* to *D4Mit13* and this set of mice is used as a control to compare with the other congenic mice groups. The genomic composition of each congenic set is illustrated in Figure 2. The *Mmom2-B* group mice carry FVB alleles from *D4Mit193* to *D4Mit13* and show a significant increase in tumor latency compared to *Mmom2-A* group mice (P=0.0096 Figure 1C). The *Mmom2-C* group mice carry FVB alleles from *D4Mit82* to *D4Mit12* and the *Mmom2-D* group mice carry FVB alleles from *D4SNP549* to *D4Mit82*. Neither of these sets of recombinant mice show a significant difference in mammary tumor latency when compared with the *Mmom2-A* group mice (P=0.89 and P=0.23, Figure 1D and 1E). This result indicates that the region from *DSNP549* to *D4Mit12* does not contain the *Mmom2* modifier. The *Mmom2-E* group mice carry FVB alleles from *D4SNP483* to *D4Mit12* and are significantly different from *Mmom2-A* group with respect to mammary tumor latency (P=0.037 Figure 1F), suggesting the *Mmom2-E* region contains the *Mmom2* modifier. We did not detect an effect on tumor number for any of the sets of congenic mice (data not shown). We were unable to identify additional polymorphic markers in the region to further define the recombination end points. Therefore, the most likely region encompassing *Mmom2* is from *D4Mit286* to *D4SNP549*, approximately 15 *cM* or 12 Mb.

Figure 1 Kaplan-Meier Analysis of tumor latency. 1A-B) Single marker analysis using *D4Mit82* and *D4SNP482*. 1C-F) Analysis of different recombinants.

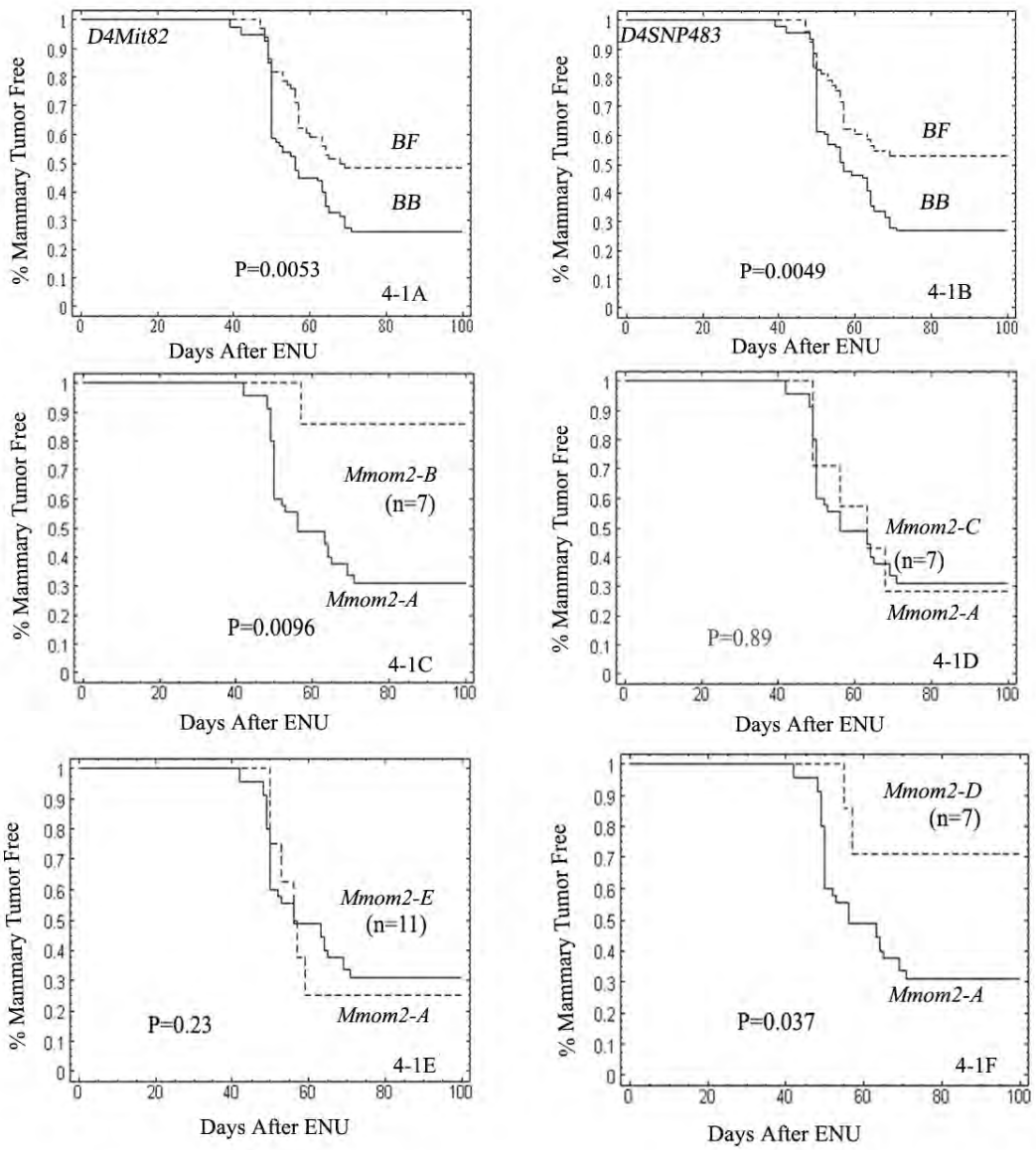


Figure 2 *Mmom2* localizes to a 12 Mb region. A black bar means the DNA sequence from B6 and a blue bar means the DNA sequence from FVB. A yellow bar means the source of DNA sequence is unknown. A red bar means the region could contain a *Mmom2* modifier gene.

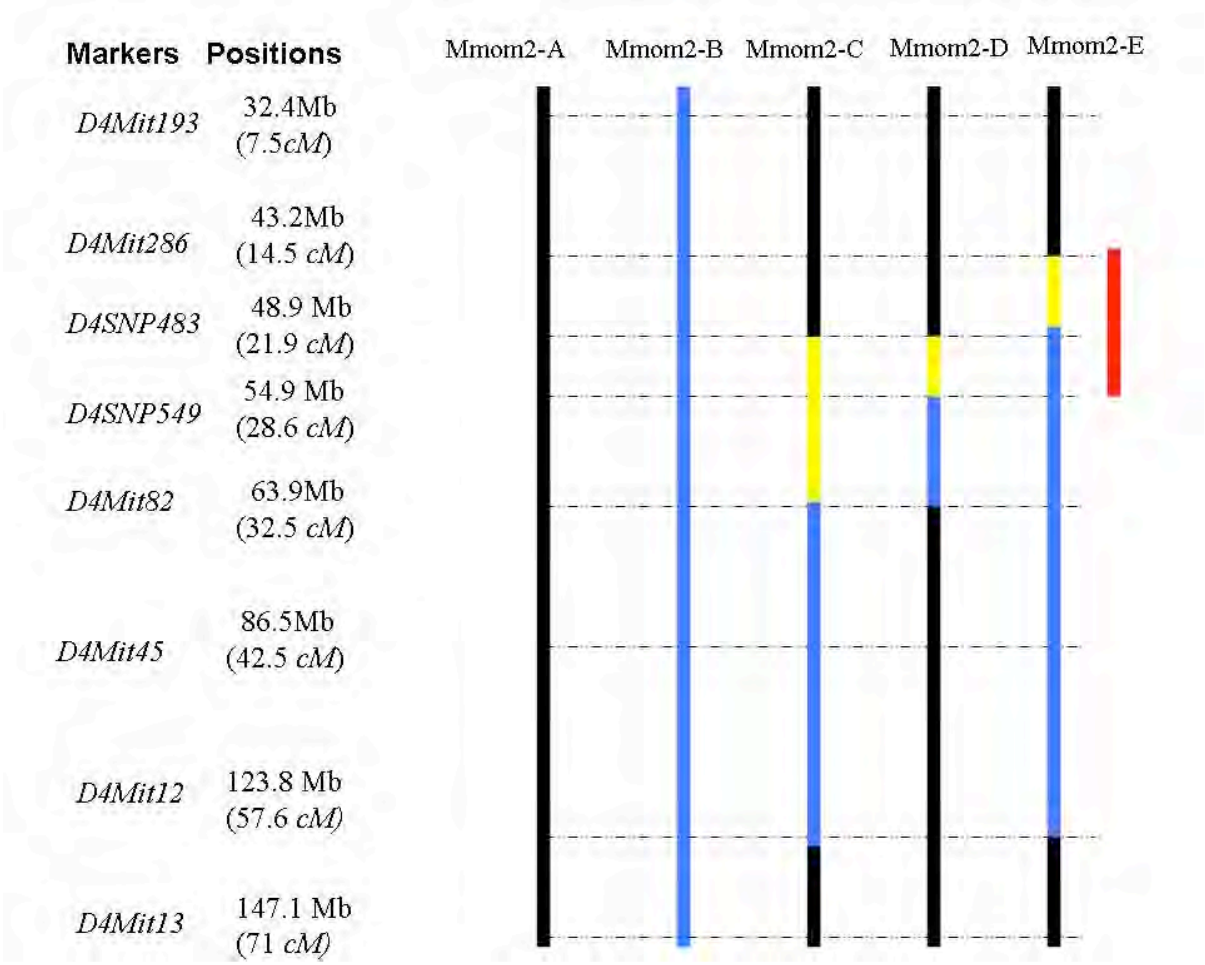


Table 1 Single Marker Analysis of *Mmom2* Congenic Mice for Tumor Multiplicity and Latency

	Tumor Latency (50% time)			Tumor Multiplicity		
	BF (Days)	BB (Days)	P Value	BF	BB	P Value
D4Mit193	>65 (n=32)	59 (n=114)	0.099	2.97±1.51 (n=32)	3.33±1.84 (n=114)	0.36
D4Mit286	65 (n=40)	60 (n=106)	0.20	3.13±1.63 (n=40)	3.3±1.83 (n=106)	0.73
D4Mit483	>69 (n=53)	57 (n=93)	**0.0049**	3.11±1.56 (n=53)	3.33±1.89 (n=93)	0.61
D4Mit82	68 (n=66)	56 (n=80)	**0.0057**	3.08±1.63 (n=66)	3.4±1.89 (n=80)	0.34
D4Mit45	69 (n=55)	57 (n=91)	**0.044**	3.05±1.61 (n=55)	3.37±1.87 (n=91)	0.33
D4Mit12	65 (n=44)	59 (n=102)	0.15	3.11±1.57 (n=44)	3.31±1.86 (n=102)	0.69
D4Mit13	64 (n=29)	63 (n=117)	0.90	3.21±1.69 (n=29)	3.26±1.8 (n=117)	0.97

B: Characterize the mechanisms of mammary modifiers.

To evaluate the combinational effect of these four different modifiers, FVB.B6-*Apc*$^{Min/+}$ congenic mice were produced and analyzed. In these mice, the mammary squamous cell carcinomas, which are frequent in the B6 *Apc*$^{Min/+}$ mice, are fully suppressed,. The FVB.B6- *Apc*$^{Min/+}$ mice develop mammary adenocarcinomas after ENU treatment, a histologically different mammary tumor type than those in the B6 *Apc*$^{Min/+}$ mice. Further characterization shows that nuclear accumulation of β-catenin is present in preneoplastic lesions, squamous metaplasias from B6 *Apc*$^{Min/+}$ mice, and focal aveolar hyperplasias from FVB.B6-*Apc*$^{Min/+}$ mice. This suggests that ENU treatment induces mutations in the wild type allele of *Apc* and activates the canonical Wnt signaling pathway. Membrane E-cadherin and β-catenin staining is lost in the squamous cell carcinomas, but still present in the adenocarcinomas, suggesting that the E-cadherin/β-catenin complex is involved in the mammary tumor fate decision. A manuscript describing these results is prepared and ready to submit (See attachments).

C: Identify genes during development of mammary tumors through microarray analysis.

This part is still pending and will be a part in a newly funded RO1 Grant (to Dr. Amy Moser)

Key Research Accomplishments

1) Four mammary modifiers have been identified to affect mammary tumor number or tumor latency and a paper was published on Cancer Research.
2) Production of chromsome 4 and chromosome 9 congenic mice
3) Priduction of FVB congenic mice.
4) Congenic mice analysis confirmed the effect of Mmom2 on tumor latency and narrowed to a 12 cM region
5) Genetic background can change the mammary tumor types in the $Apc^{Min/+}$ mice

Reportable Outcomes

1) Meeting and Abstract

Genetic Dissection of mammary tumor development in *Apc*$^{Min/+}$ mice. 20th Mammalian Genome conference, Charleston, SC Nov 12-16.

The effect of genetic background on tumor development in *Apc*$^{Min/+}$ mice. Building a Better Mouse (BBM) II conference on July 12-14, 2007 at Vanderbilt University in Nashville, Tennessee.

FVB- *Apc*$^{Min/+}$: a New Mice Model to Study Proximal Colon Tumor. AACR colon cancer meeting, Hyatt Regency Cambridge, Massachusetts

2) Publication

Identification of Novel Modifier Loci of *Apc*^{Min} Affecting Mammary Tumor Development Hua Wang, Douglas Teske, Alyssa Tess, Rebecca Kohlhepp, YounJeong Choi, Christina Kendziorski, and Amy Rapaich Moser Cancer Res 2007 67: 11226-11233

Genetic background can affect mammary tumor types Hua Wang, Ruth Sullivan, Amy R Moser (In preparation)

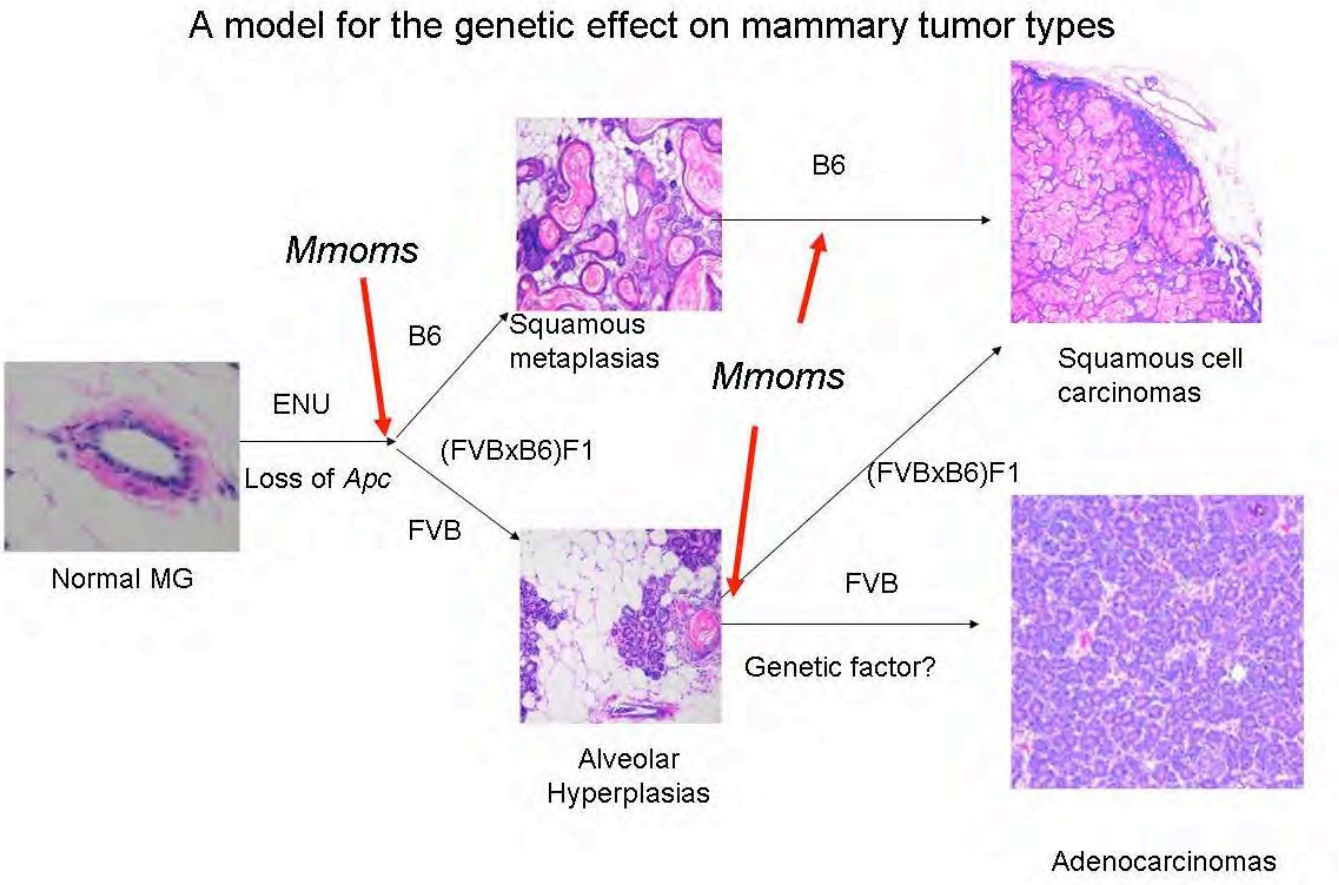
Identification of Modifier Loci Affecting Intestinal Tumor Location in *Apc*$^{Min/+}$ mice. Hua Wang, Jeena Choi, Amy R Moser (in preparation)

3) The Recipient of this grant (Hua Wang) has finished PH.D study and will be awarded with PhD degree in the May 2008. Hua Wang will continue to work on breast cancer in David Livingston's lab at Dana-Farber Cancer Institute.

4) A new RO1 grant was funded to Dr. Amy Moser to continue work on the mammary modifiers (Most preliminary results are generated during the training period of Hua Wang)

Conclusion

Four mammary modifiers of Min have been identified and one modifier was narrowed into a 12cM region. We provide evidence to support that genetic background can affect tumor types. A model for the genetic effect on mammary tumor types is proposed. The ENU treatment would induce the loss of wild type allele of *Apc* and activate the canonical Wnt signaling pathway. In cells with loss of *Apc* function, normal mammary cells could develop into squamous metaplasias on the B6 background or focal alveolar hyperplasias on the F1 or FVB background. The squamous metaplasias would develop rapidly into SCC in B6 mice. The alveolar hyperplsias would develop to SCC in F1 mice with increased latency or adenocarcinomas in FVB mice. The process could be regulated by different factors. Some of the Mammary modifiers of Min could affect which preneoplastic lesions arise; squamous metaplasias or focal alverolar hyperplasias. Some of these modifiers could affect the progression of squamous metaplasias to SCC or the growth of SCC. Other modifiers could affect which tumor types could arise from focal alveolar hyperplasias.



Identification of Novel Modifier Loci of *Apc*^{Min} Affecting Mammary Tumor Development

Hua Wang,[1,3] Douglas Teske,[1] Alyssa Tess,[1] Rebecca Kohlhepp,[1] YounJeong Choi,[2] Christina Kendziorski,[2] and Amy Rapaich Moser[1]

Departments of [1]Human Oncology and [2]Biostatistics and Medical Informatics, School of Medicine and Public Health and [3]Department of Genetics, University of Wisconsin-Madison, Madison, Wisconsin

Abstract

Genetic background affects the susceptibility to mammary tumor development in *Apc*$^{Min/+}$ mice. Here we report the identification of four novel modifier loci that influence different aspects of mammary tumor development in *Apc*$^{Min/+}$ mice. Analysis of tumor development in a backcross of (FVBB6 *Apc*$^{Min/+}$) × B6 *Apc*$^{Min/+}$ mice has identified a modifier on chromosome 9 that significantly affects tumor multiplicity, and a modifier on chromosome 4 that significantly affects tumor latency and affects tumor number with suggestive significance. This modifier was also identified in a backcross involving 129X1/SvJ and B6 *Apc*$^{Min/+}$ mice. A modifier on chromosome 18 specifically affects tumor latency but not tumor number. Kaplan-Meier analysis suggests there is at least an additive interaction affecting tumor latency between the loci on chromosomes 4 and 18. We also identified a modifier locus on chromosome 6 that interacts with the loci on chromosome 4 and chromosome 9 to affect tumor number. These results suggest that multiple genetic loci control different aspects of mammary tumor development. None of these modifiers is associated with intestinal tumor susceptibility, which indicates that these modifiers act on tumor development in a tissue-specific manner. [Cancer Res 2007;67(23):11226–33]

Introduction

Breast cancer develops through multiple steps that are rigorously controlled by genetic factors (1). It is essential to identify and characterize genes controlling the development of breast cancer to better understand factors affecting tumor susceptibility and contribute to better diagnosis and treatment. Advances have been made toward identifying common alleles of genes that affect breast cancer susceptibility in humans, using single nucleotide polymorphisms (SNP) and very large sample sizes (2). The risk associated with each of these common variants is small, and such studies have the little power to detect low frequency variants. Mouse models provide useful tools to identify modifiers of cancer with major or minor effect (3). As many of the genes known to be involved in human breast cancer also affect mammary tumor development in mice, modifier genes identified in mice are likely to also play a role in regulating the development of breast cancer in humans.

The transition from hyperplastic lesions to tumors is critical for the development of cancers (4). In the human, ductal and lobular hyperplasias are known risk factors for cancer development. For example, in one study, about 20% of women diagnosed with atypical lobular hyperplasia or lobular carcinoma *in situ* later developed cancer (5). Interestingly, this risk for cancer development applies to both the involved and contralateral breast in these women (5). This transition is poorly understood due to both the difficulty of identifying and tracking early hyperplastic lesions and the lack of good mouse models.

The *Apc*$^{Min/+}$ mouse is a well-characterized mouse model that is predisposed to both intestinal and mammary tumors (6). Upon exposure to the alkylating agent ethylnitrosourea (ENU), >90% of B6-*Apc*$^{Min/+}$ female mice develop mammary squamous cell carcinomas (SCC) within 65 days, with an average 3.3 tumors per mouse and an average latency of about 56 days. FVBB6 F1 *Apc*$^{Min/+}$ mice are resistant to mammary tumors but susceptible to focal alveolar hyperplasias under the same treatment (7). This suggests that FVB carries alleles at modifier loci of *Apc*^{Min} that act dominantly to affect the progression of hyperplasias to tumors. The identification of such modifiers could provide biomarkers of hyperplasias that could be helpful in the early diagnosis of breast cancer.

The inheritance of a mutation in *APC* gives predisposition to the autosomal dominant disorder familial adenomatous polyposis in humans (8, 9). Mouse APC shares 90% amino acid identity with human APC, and germline mutations result in similar phenotypes. *Min*, a dominant allele of *Apc* in the mouse, was generated by ENU germline mutagenesis and results in a truncation mutation at codon 850 (10). The insight that genetic background affects intestinal tumor development led to the mapping and molecular identification of *Mom1* (modifier of Min 1; refs. 11, 12).

Wnt signaling has been observed to be abnormally activated in many cancers including breast cancer in both mice and humans. APC is critically important in the canonical Wnt signaling pathway and, together with GSK-3β and Axin, can participate in the phosphorylation of β-catenin, which is then targeted for degradation (13, 14). Upon Wnt activation or mutations of other components, β-catenin can translocate into the nucleus and bind with transcription factors, such as TCF4, to activate target genes including *cylin D1* and *c-myc* (13, 14). Transgenic expression of β-catenin, cyclin D1, and c-myc in mammary tissue leads to mammary tumors in mice (15–17). Amplification of *cyclin D1* was found in >50% human breast cancer, and somatic *APC* mutations were found in 18% breast cancer (18–20). The aberrant regulation of β-catenin is a prognostic marker in human breast cancer (21, 22). Overall, these data provide strong evidence that activation of canonic Wnt signaling is a common feature in human breast cancer.

In B6 *Apc*$^{Min/+}$ female mice, SCC develop rapidly after ENU treatment with nearly all of the mice developing tumors by 65 days after treatment. In contrast, when treated with ENU, FVBxB6

Requests for reprints: Amy R. Moser, Department of Human Oncology, University of Wisconsin-Madison, K4/310 CSC Box 3684, 600 Highland Avenue, Madison, WI 53792. Phone: 608-265-6520; Fax: 608-263-9947; E-mail: armoser@wisc.edu.

doi:10.1158/0008-5472.CAN-07-2487

$Apc^{Min/+}$ female mice develop multiple alveolar hyperplasias and few SCC with long latency (7). Because the initiation step is most likely the same in both of these strains of mice, loss of the wild-type allele of *Apc* due to ENU-induced mutation, the difference in mammary tumor susceptibility must be due to genes that affect the process of tumor development. The resistance to mammary tumor development in FVBB6 F1 $Apc^{Min/+}$ females could be due to the effect of modifier loci on any of the steps of tumor development. However, the susceptibility of FVBB6 F1 $Apc^{Min/+}$ females to hyperplasias suggests the modifier loci do not affect the initiation of hyperplasia. Thus, the modifier loci most likely affect the transition of hyperplasias to mammary tumors or the progression and growth of mammary tumors. As a first step toward understanding the genetic control of mammary tumor development in these mice, we performed backcross analysis, mapping the traits of tumor number and tumor latency. Further molecular identification of these modifier genes would provide more insights into the regulation of the Wnt signaling pathway and mechanisms of breast cancer development.

Materials and Methods

Animals and phenotypes. All mice were maintained in the Laboratory Animal Research Facility of University of Wisconsin School of Medicine and Public Health. All procedures were approved by the Institutional Animal Care and Use Committee. Female C57BL/6J (B6), 129X1/SvJ (129X1), and FVB/NTac (FVB) mice were purchased from The Jackson Laboratory or Taconic Labs. C57BL/6J $Apc^{Min/+}$ (B6 $Apc^{Min/+}$) mice are maintained by crossing wild-type B6 females from The Jackson Laboratory with B6 $Apc^{Min/+}$ males. The males used in these experiments were from generations N35-N66.

For the FVBB6 backcross, wild-type FVB females were mated to B6 $Apc^{Min/+}$ males (N59-66) to produce F1 females. The F1 $Apc^{+/+}$ females (n = 72) were backcrossed with B6 $Apc^{Min/+}$ males (n = 48) to produce N2 females. A total of 307 $Apc^{Min/+}$ N2 females were produced and treated with 50 mg/kg ENU by i.p. injection when between 35 to 40 days of age and were euthanized ~75 days after ENU treatment as described (7). Mice were palpated weekly by the same observer (D. Teske), and palpable tumors were recorded when first noted and then confirmed at necropsy. The total tumor number was determined at necropsy by noting all visible discrete masses. Tumors were collected and fixed in formalin for histologic analysis. We also collected 4-cm regions from the proximal, middle, and distal small intestine and the whole colon for counting intestinal tumors.

For the 129X1B6 backcross, wild-type 129X1 female mice (N = 42) were crossed to B6 $Apc^{Min/+}$ males (N35-36; n = 7) to produce (129X1B6) F1 mice. Backcross mice were produced by crossing (129X1B6) F1 females to B6 $Apc^{Min/+}$ males (n = 7), (129X1B6) F1 $Apc^{Min/+}$ females to B6 male mice (n = 18), and B6 females to (129X1B6) F1 $Apc^{Min/+}$ male mice (n = 55). A total of 80 129X1B6 backcross $Apc^{Min/+}$ females were produced. The mice were ENU-treated and followed as described above for the FVB backcross.

Genotyping. For $Apc^{Min/+}$ genotyping, genomic DNA was obtained after incubating tissues in 100 μL of 50 mmol/L NaOH at 95°C for 1 h and then adding 10 μL of 1 mol/L Tris (pH = 5), modified from the previous report (23). Animals were genotyped for $Apc^{Min/+}$ by PCR using an allele-specific PCR assay (11).

For both backcross analyses, DNA was isolated from the spleens of backcross females using the DNeasy kit (Qiagen). Simple sequence length polymorphism (SSLP) primers were purchased from Research Genetics or Integrated DNA Technologies. For the FVBxB6 BC, a total of 102 SSLP markers with an average spacing of about 20 cM were genotyped for 180 mice selected from the ends of the tumor number distribution (90 mice with 1 or fewer tumors and 90 with 3 or more). Chromosomes with putative modifier loci were then genotyped with higher density markers for the remaining 127 mice. For the 129 backcross, all 80 mice were genotyped with 79 SSLP markers. Markers are available upon request. The PCR reactions were set up with 20 μL containing 50 to 100 ng genomic DNA, 2.0 μL 10 × buffer, 1.5 μL 25 nmol/L $MgCl_2$, 30 nmol/L forward and reverse primers, and 0.5 μL 10 nmol/L deoxynucleotide triphosphates. The PCR conditions were 2 min at 94°C for 1 cycle; 15 s at 94°C, 45 s at 55°C, and 45 s at 72°C for 35 cycles; and finally, 7 min at 72° for 1 cycle. PCR products were resolved on 4% agarose gels and visualized by ethidium bromide staining.

Statistical analysis. The number of QTL controlling mammary tumor number was estimated as described in Dietrich et al. (11) with the classic formula of Wright, $n = (u_{F1}\text{-}u_P)^2/4\sigma_G^2$. Usually this formula underestimates the number of QTL because the assumptions are not exactly satisfied. MSTAT, a program provided by Dr. Norman Drinkwater of the McArdle Laboratory at the University of Wisconsin-Madison (Madison, WI), was used for the Kaplan-Meier analysis of tumor latency.

Linkage analysis was carried out using the parametric and nonparametric methods implemented in R/qtl (ref. 24; referred to hereinafter as R/qtl-P and R/qtl-NP, respectively) and the nonparametric methods implemented in the Q-link program, provided by Dr. Drinkwater. R/qtl-P implements a hidden Markov model to deal with missing genotypes; the expectation-maximization algorithm was used to perform single QTL genome scans (24). For Q-link, the statistic Zw is determined for each of the markers using the Wilcoxon rank-sum test and a logarithm of odds (LOD) score was calculated by the function $LOD_W = 0.5\ (\log_{10} e)\ (Z_W)$ (ref. 25); R/qtl-NP also uses a Wilcoxon rank-sum test but allows for LOD score evaluation between markers (24). For the linkage analyses of tumor latency, nonparametric methods were used because the raw and transformed phenotypes were not Gaussian. Permutation tests were performed (10,000 permutations per phenotype) to determine thresholds for suggestive ($P < 0.05$) and significant linkage ($P < 0.01$) at the genome level.

Two methods were used to assess the presence of interactions among QTL. We first considered a model selection procedure as detailed in Lan et al. (26). That procedure identifies potential interactions among QTL and other genome regions that perhaps do not show significant main effects. In short, the procedure first identifies putative QTL using a LOD score profile. In the initial step, we do not require that putative QTL be statistically significant; they are defined as those with LODs >1, where LOD scores are calculated using R/qtl-P. We then consider all possible models allowing for additive effects among the putative QTL and pairwise interactions. The Bayes Information Criterion (BIC; ref. 27) is used to score each model. The BIC balances goodness-of-model fit with the number of model variables. The model with the best (lowest) BIC is then identified. We also considered the two dimensional scans provided by R/qtl. There, each pair of loci is evaluated allowing for main effects and the pairwise interaction between those effects. The likelihood of the full model is then compared with alternatives to test for additive and interaction effects (24).

To combine the FVBB6 and 129X1B6 backcrosses (28), the FVB and 129X1 alleles were assigned the same genotype and the B6 allele was treated as the other genotype. Therefore, a binary allelic pattern was used, and only loci where both FVB and 129X1 carry alleles that affect tumor development in the same direction will be identified as modifiers.

Results

Tumor number and tumor latency in the FVB backcross. To map modifier loci affecting the development of mammary tumors, 307 backcross $Apc^{Min/+}$ females were treated with ENU (7). Approximately 90% of the backcross $Apc^{Min/+}$ female mice developed visible mammary tumors with an average of 2.54 tumors per mouse at necropsy. More than 400 mammary tumors from the first 200 mice were examined histologically, and all were classified as SCC (data not shown). With the classic Wrights formula, it was estimated that 1.3 genetic loci control tumor number in this backcross.

By the end of the experiment, 57% of backcross females had developed at least one palpable tumor (meaning that the tumor was detected before necropsy). The classification of mammary

tumors as palpable tumors is dependent on both tumor size and location. However, because some tumors did develop in each of the mammary glands in the set of backcross mice, it is expected that tumors in all mice had approximately equal chances of detection. Among a total of 780 visible mammary tumors in the backcross females, about one third (259 of 780) were palpable and two thirds (521 of 780) were detected only at dissection. On average, the backcross $Apc^{Min/+}$ females developed 0.84 palpable tumors and 1.70 nonpalpable tumors.

Identification of modifiers affecting tumor number in the FVBB6 backcross. A whole genome scan with 102 SSLP markers spaced an average of 20 cM apart was performed with mice at the two extremes of the tumor number distribution: 90 mice with 0 or 1 tumor and 90 mice with <3 tumors (Fig. 1*A*). The rest of the mice were then genotyped only on chromosomes with a LOD score of >1. Three loci, on chromosomes 4, 6, and 9, were associated with mammary tumor number. These QTL are designated *Mmom1* (mammary modifier of *Min*; chromosome 9), *Mmom2* (chromosome 4), and *Mmom3* (chromosome 6) in order of effect. The summary of the QTL analysis is shown in Table 1, and the LOD profile for each chromosome is shown in Fig. 1*B*.

As shown in Tables 1 and 2, both the Q-link and R/qtl-P analysis methods gave comparable results, although they adopt different models for analysis. *Mmom1,* associated with *D9Mit182* (55 cM), reaches statistical significance for an effect on tumor multiplicity. *Mmom1* heterozygous females show about a 30% decrease in tumor number compared with homozygous mice. *Mmom2,* on chromosome 4 at *D4Mit82* (32.5 cM), shows suggestive significance for an effect on tumor number. *Mmom2* heterozygous females developed 24% fewer mammary tumors than homozygous mice.

The modifier on chromosome 6 at *D6Mit31* (38.5 cM) does not reach significance, but the approach of Lan (26) applied to the

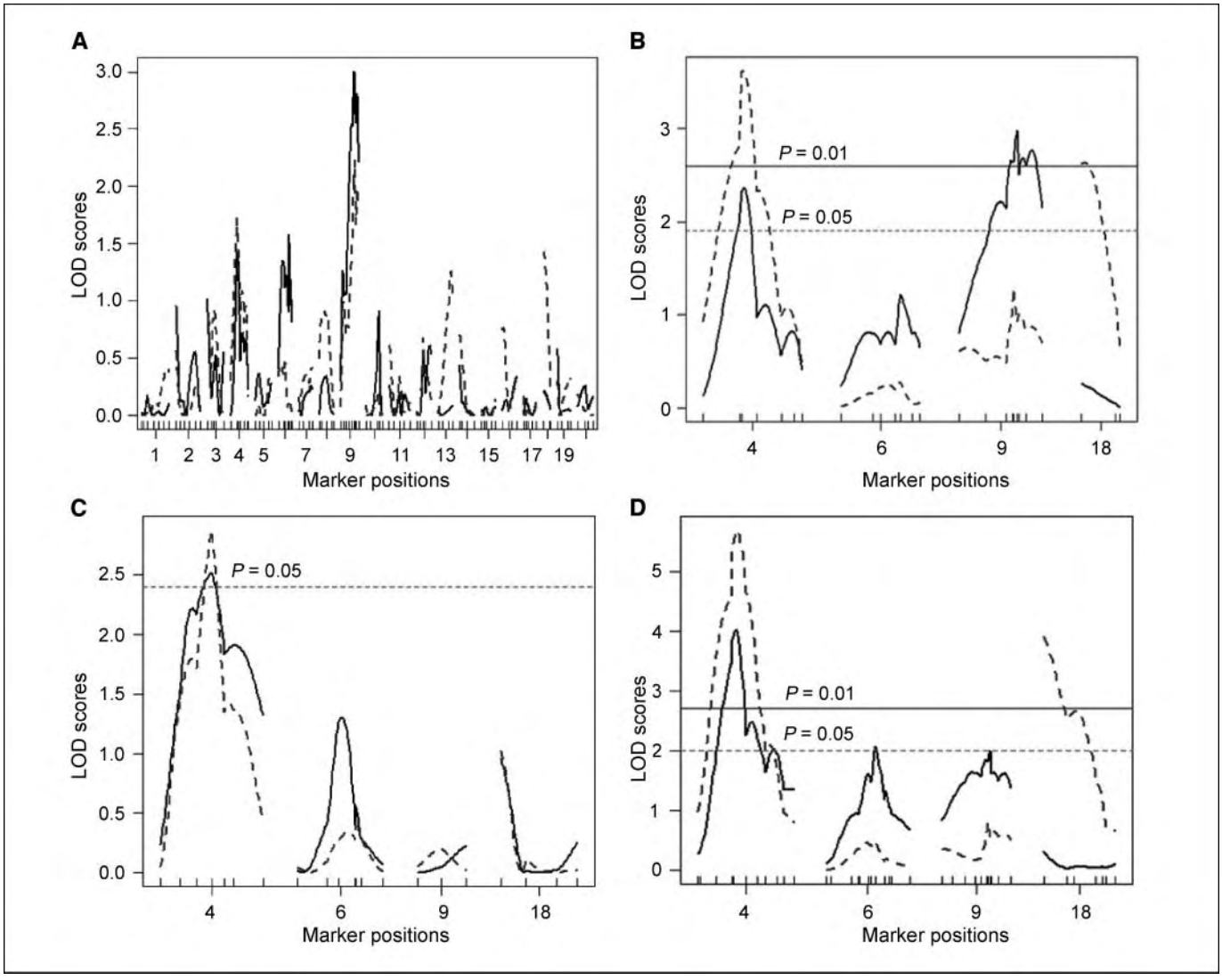


Figure 1. The LOD score profile of mammary modifiers of $Apc^{Min/+}$. The tumor numbers were analyzed by the parametric method, and the tumor latency was analyzed by the nonparametric method in R/qtl. *Solid lines,* the result of tumor number analyses; *dashed lines,* the result of tumor latency analyses. *B* and *D,* the suggestive or significant levels; *C,* the suggestive level. *A,* the LOD profile of tumor number and tumor latency with 180 selectively genotyped mice from the FVBB6 backcross. *B,* the LOD profile on the selected chromosomes for tumor number and tumor latency from the entire FVBB6 backcross. *C,* the LOD profile on the selected chromosomes for tumor number and tumor latency from 129X1B6 backcross. *D,* the LOD profile of the modifier loci for tumor number and tumor latency analysis from the combined FVBB6 and 129X1B6 crosses.

Table 1. Summary of the effect of modifier loci on tumor multiplicity and tumor latency using Q-link

Locus	Peak marker	Tumor multiplicity*			Tumor latency*		
		LOD score	Average tumor no. (no. mice)		LOD score	50% time †	
		Q-link	BB ‡	BF ‡	Q-link	BB ‡ (d)	BF ‡ (d)
FVBB6 Backcross							
Mmom1	*D9Mit182*	3.1§	2.9 ± 1.9 (n = 143)	2.1 ± 1.8 (n = 163)	1.0	63	71
Mmom2	*D4Mit82*	2.1‖	2.9 ± 2.0 (n = 147)	2.2 ± 1.7 (n = 159)	3.5§	57	>78
Mmom3	*D6Mit36*	1.1	2.8 ± 2.0 (n = 165)	2.3 ± 1.7 (n = 142)	0.2	65	73
Mmom4	*D18Mit24*	0.2	2.7 ± 1.9 (n = 161)	2.4 ± 1.9 (n = 146)	2.6§	59	>78
129X1B6 Backcross			BB ‡	B129 ‡		BB ‡	B129 ‡
Mmom2¶	*D4Mit26*	2.4‖	2.8 ± 2.1 (n = 44)	1.3 ± 1.3 (n = 34)	3.0§	54	>99

*For tumor multiplicity, the LOD thresholds of P = 0.05 and P = 0.01 are 1.93 and 2.64. For tumor latency, the LOD thresholds of P = 0.05 and P = 0.01 are 1.9 and 2.6, as determined by 10,000 permutation tests in Q-link.
†50% time means the time when >50% mice developed palpable mammary tumors.
‡ BB, homozygous for B6 alleles; BF, heterozygous for B6 and FVB alleles; B129, heterozgous for B6 and 129 alleles.
§ P < = 0.01.
‖ P < 0.05.
¶The threshold of P = 0.05 in 129B6 backcross are 2.39 for tumor number and 2.43 for tumor latency.

FVBB6 backcross identified an additive model with *Mmom1, Mmom2*, and *Mmom3*. Accounting for interactions did not improve the BIC (data not shown). These results are consistent with the joint scans provided by R/qtl. Using that approach, the joint LOD score between *Mmom1* and *Mmom3* reaches significance (LOD = 4.46; P < 0.01), providing further evidence of the additive effects of these two loci. The effects are shown explicitly in Fig. 2*A*. When mice are heterozygous at both *Mmom1* and *Mmom3*, tumor number is reduced ~50% compared with mice homozygous at both loci (Fig. 2*A*; 1.7 ± 1.5 versus 3.2 ± 2.0). *Mmom3* also has an additive effect in concert with *Mmom2* (Fig. 2*B*; 3.3 ± 2.2 versus 2.0 ± 1.6). Taken together, these analyses indicate that although the locus on chromosome 6 fails to achieve significance in the one-dimensional scans, this locus does influence tumor number in this backcross.

Identification of modifiers associated with tumor latency in the FVBB6 backcross. The number of mammary tumors observed at necropsy reflects the combination of multiple events including lesion initiation, hyperplasia growth, transition of hyperplasias, tumor progression, and tumor growth. Using nonparametric methods, we analyzed the FVBB6 backcross to search for genetic loci controlling the time to tumor (tumor latency). Time to tumor was defined as the time of appearance of the first palpable tumor after ENU treatment. All mice without palpable tumors at necropsy were assigned the highest rank order.

A whole genome scan was carried out as with tumor multiplicity (using the mice on the ends of the tumor number distribution) and chromosomes with LOD > 1 were selected for further genotyping and analysis using all of the mice. Modifier loci on chromosomes 4 and 18 were found to significantly affect tumor latency (Fig. 1*B* and Table 1). The modifiers on chromosomes 6 and 9 did not show a significant effect on tumor latency. The peak position of the QTL

Table 2. The combined analysis of modifiers of *Apc*$^{Min/+}$ from FVBBC and 129B6 backcross using R/qtl

Loci	Tumor multiplicity LOD			Tumor latency LOD		
	FVBB6	129B6	Combined	FVBB6	129B6	Combined
Mmom1	3.0*	0.2	1.91	1.3	0.20	0.8
Mmom2	2.3 †	2.5 †	3.99 ‡	3.6*	2.9*	5.7 ‡
Mmom3	1.2	1.3	2.08 †	0.3	0.4	0.5
Mmom4	0.3	1.0	0.31	2.6*	1.0	3.9 ‡

NOTE: The threshold of P = 0.05, P = 0.01, and P = 0.001 for tumor multiplicity are 1.99, 2.70, and 3.67 with R/qtl-P. The threshold of P = 0.05 and P = 0.01 for tumor multiplicity are 1.98, 2.69, and 3.68 with R/qtl-NP.
* P < 0.01.
† P < 0.05.
‡ P < 0.001.

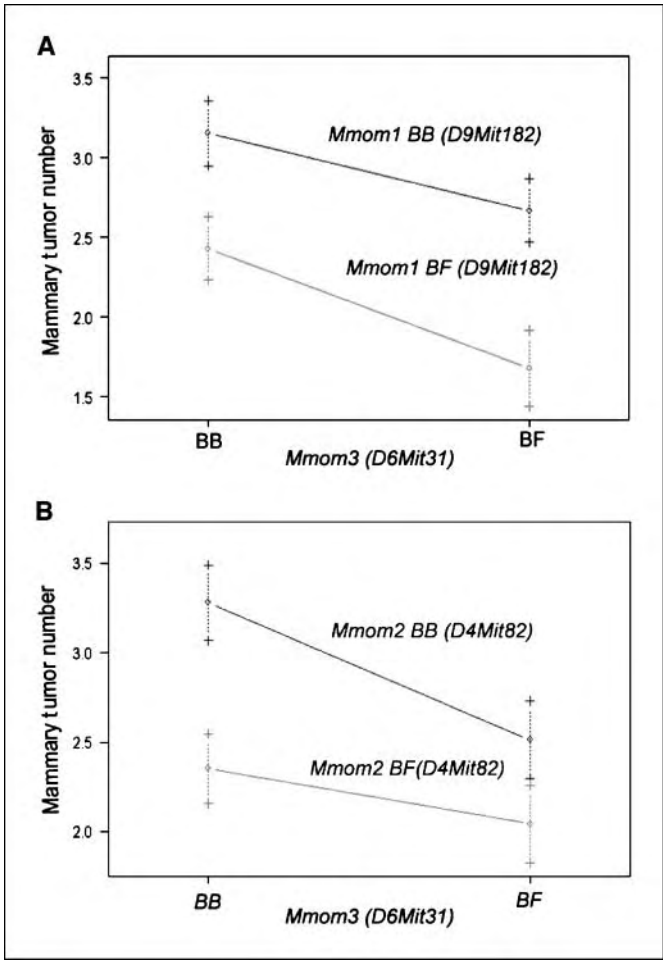


Figure 2. Effect of *Mmom1, Mmom2*, and *Mmom3* on mammary tumor number in the FVBB6 Backcross. *A,* additive effects of *Mmom1* and *Mmom3*. *B,* additive effects of *Mmom2* and *Mmom3*.

affecting latency is the same as the QTL affecting tumor number on chromosome 4 (*D4mit82* and *Mmom2*). This suggests that *Mmom2* could affect both tumor number and tumor latency. Less than 50% of mice carrying an FVB allele at *Mmom2* developed a palpable mammary tumor by the end of experiment (75 days after ENU), whereas 50% of mice homozygous for B6 alleles had palpable tumors by 57 days after ENU treatment. A locus on chromosome 18 at *D18Mit24* (25 cM) specifically affects tumor latency and was designated *Mmom4*. This locus shows no effect on the tumor multiplicity. This locus maps about 10 cM distal of the *Apc* locus, which also maps to chromosome 18.

To determine the effect of each modifier on tumor development, we performed Kaplan-Meier analysis stratifying the mice by genotype at *D4Mit82* or *D18Mit24*. The tumor latency for the mice homozygous at each marker is significantly different from the tumor latency for the mice heterozygous at each marker (Fig. 3*A* and *C*). We then stratified for both markers and found additive effects between *Mmom2* and *Mmom4* (Fig. 3*D*). When mice were homozygous for B6 alleles at both loci, around 80% of females developed a palpable tumor. When the mice were heterozygous at both loci, only 40% of the mice developed a palpable tumor ($P < 0.001$). Heterozygosity for either one of the loci has approximately the same effect on tumor latency.

Identification of *Mmom2* in the 129X1B6 backcross. The identification of *Mmom2* is supported by an independent backcross involving 129X1 and B6 $Apc^{Min/+}$ mice. A locus that reaches suggestive significance for an effect on tumor number maps to the same region of chromosome 4 as *Mmom2* (Fig. 1*C* and Table 1). We then tested for an effect on tumor latency. A marker, *D4Mit26*, which maps about 10 cM distal to *D4Mit82*, reaches significance for an effect on tumor latency (Fig. 1*C* and Table 1). The Kaplan-Meier analysis also shows that tumor latency of the *D4Mit26* heterozygous mice is significantly different from the homozygous mice (Fig. 2*B*). Because the modifiers identified in the 129B6 backcross and the FVBB6 backcross affect the same phenotype, and the two peak markers, *D4Mit82* and *D4Mit26*, map close to each other, they most likely represent the same modifier QTL, *Mmom2.* In this backcross, we also found that a potential modifier affecting tumor number mapped to the region of *Mmom3* on chromosome 6 and a potential modifier affecting both tumor latency and tumor number mapped to the same position as *Mmom4* on chromosome 18, but neither locus reached significance (Fig. 1*C* and Table 2).

As the data from both the FVBB6 and the 129B6 backcrosses were collected in a similar way, we combined these two backcrosses to improve the power of modifier mapping (28). Because different markers are used in these two backcrosses, 1-cM interval mapping was used to calculate the LOD score for each modifier with the software R/qtl. The hypothesis is that if a modifier is shared between different crosses, the combined cross can improve the LOD score and would dilute the effect of cross-specific modifier QTL. Figure 1D shows the LOD profile in the combined analysis and the results are summarized in Table 2. On chromosome 4, the LOD score in the region of *Mmom2* was highly significant ($P < 0.001$) for both tumor number and tumor latency. This suggests that the resistant allele of *Mmom2* is shared by the FVB and 129X1 strains. On chromosome 6, the LOD score of *Mmom3* reached suggestive significance ($P < 0.05$) for tumor number with no effect on tumor latency. This provides more evidence that there is a shared modifier on chromosome 6 affecting tumor number. On chromosome 9, the LOD score of *Mmom1* decreased compared with FVBB6 backcross and there was no effect on tumor latency. This suggests that the 129X1 and B6 strains share alleles with similar effect at *Mmom1*. On chromosome 18, the LOD score of *Mmom4* was highly significant ($P < 0.001$) for tumor latency, indicating that FVB and 129X1 strains share alleles at *Mmom4*. In summary, the combined cross analysis confirmed the results of individual backcross analyses and provided more support for the effect of *Mmom2, Mmom3,* and *Mmom4* on tumor development.

Tissue specificity of modifiers. Because $Apc^{Min/+}$ mice develop both mammary and intestinal tumors, we could test for the effect of any modifiers on both tumor types. None of the *Mmom* loci affected intestinal tumor development (Table 3). *D4Mit13* (71 cM), the most distal marker on chromosome 4 in the FVBB6 backcross, is significantly associated with intestinal tumor number but has no effect on mammary tumor number and latency. *D4Mit13* maps to the same position as *Mom1* (11). This suggests that the FVB strain carries a resistant allele of *Mom1* (11). A database search identified a SNP (*rs27560348*, T/C, and dbSNP126) between B6 and FVB in the phospholipase gene *Pla2g2a*. This SNP causes a frameshift mutation and generates a mutant allele for *Pla2g2a* in B6 mice (12). Taken

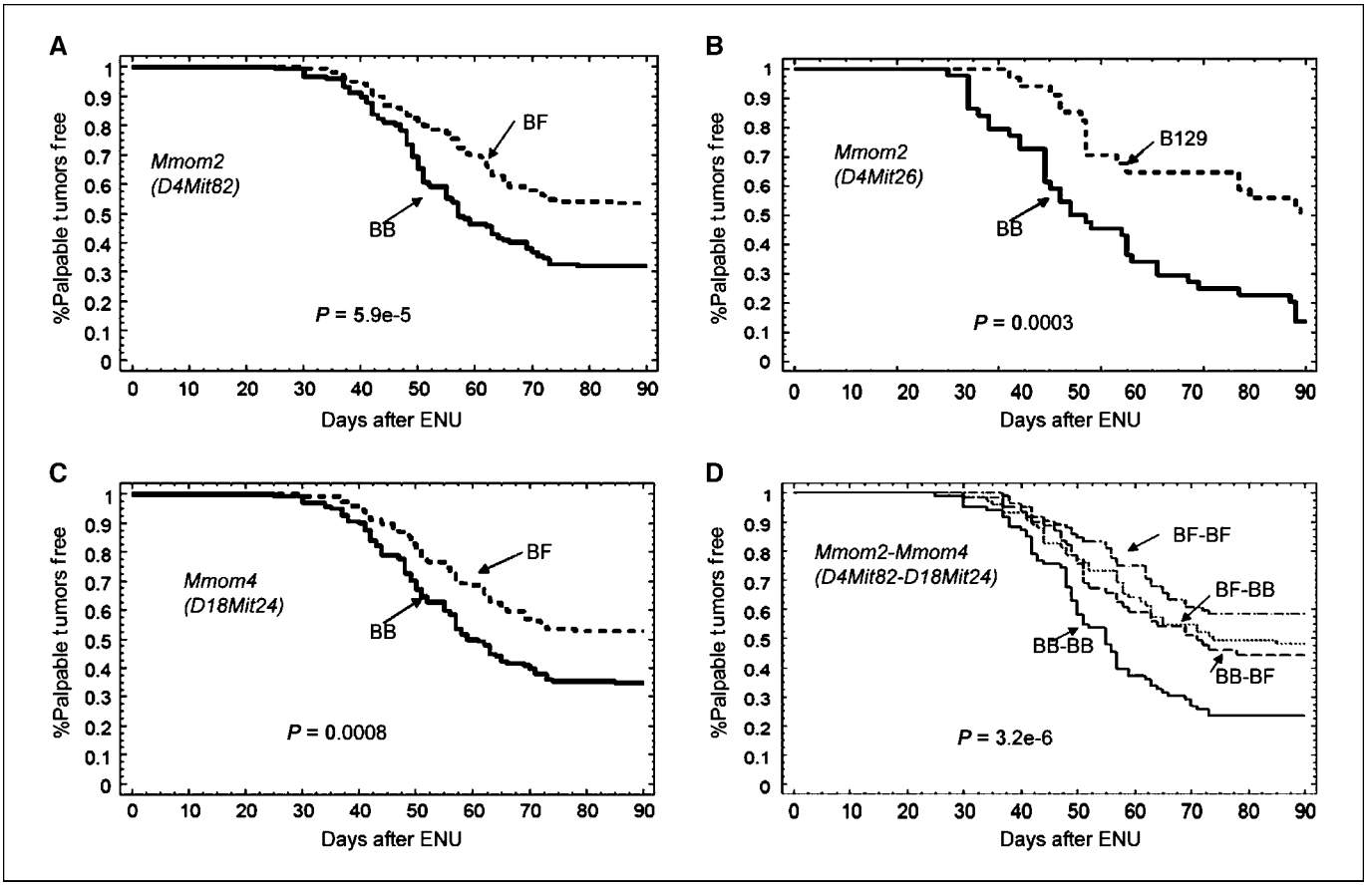


Figure 3. Kaplan-Meier analyses of the effect of modifiers of $Apc^{Min/+}$ on tumor latency. The *P* values indicated were determined by the log-rank test. *BB,* B6 homozygous; *BF,* B6 and FVB heterozygous; B129 means B6 and 129X1 heterozygous. *A,* the effect of *Mmom2* on tumor latency in the FVBB6 backcross. *B,* the effect of *Mmom2* on tumor latency in the 129X1B6 backcross. *C,* the effect of *Mmom4* on tumor latency in the FVBB6 backcross. *D,* the interaction of *Mmom2* and *Mmom4* on tumor latency in the FVBB6 backcross.

together, these data provide strong evidence that the modifiers of Apc^{Min} show tissue specificity for mammary tumors and intestinal tumors.

Discussion

The development of mammary tumors is a complex process that involves a series of events including initiation, the growth of hyperplasias, transition from hyperplasia to tumor, and tumor progression and growth (29, 30). To test for an effect on each stage of tumor development, we recorded the number of tumors and hyperplasias and classified each tumor and hyperplastic lesion histologically. No loci showed an association with the total number of mammary lesions, which included all tumors and hyperplastic lesions (data not shown). These results suggest that modifiers are not involved in DNA repair after ENU treatment, the initiating

Table 3. Analysis of modifiers if $Apc^{Min/+}$ affecting intestinal tumor multiplicity in the FVBB6 backcross using Q-link

				Intestinal number	
Loci	Markers	*P* value	LOD	BB	BF
Mom1	*D4Mit13*	4.0e-17*	15.4*	31.0 ± 11.9 (*n* = 120)	17.7 ± 8.9 (*n* = 120)
Mmom1	*D9Mit182*	0.09	0.6	25.7 ± 11.5 (*n* = 126)	23.6 ± 13.2 (*n* = 116)
Mmom2	*D4Mit82*	0.3	0.2	25.3 ± 12.0 (*n* = 121)	24.3 ± 12.7 (*n* = 121)
Mmom3	*D6Mit31*	0.1	0.4	26.2 ± 13.7 (*n* = 131)	23.0 ± 10.4 (*n* = 112)
Mmom4	*D18Mit24*	0.6	0.05	24.8 ± 11.4 (*n* = 134)	24.7 ± 13.5 (*n* = 109)

NOTE: The thresholds for $P = 0.05$ and $P = 0.01$ are 1.9 and 2.6. This is determined by 10,000 permutation test in Q-link.
*$P < 0.001$.

event in this model. This was not necessarily a surprising result as the number of total lesions in the B6 and FVBxB6 F1 mice is similar (7); thus, we did not expect to see an effect on lesion initiation.

In this analysis, we have identified modifier loci that affect either mammary tumor number or/and tumor latency. *Mmom1* on chromosome 9 affects only mammary tumor number, suggesting that it may affect the probability of transition from hyperplasia to tumor. *Mmom2* on chromosome 4 affects both tumor number and tumor latency, suggesting that it may affect both the probability of transition from hyperplasia to tumor and the rate of tumor growth. This effect is similar in both backcrosses investigated. *Mmom3* on chromosome 6 seems to cooperate with *Mmom1* or *Mmom2* to affect tumor number. *Mmom4* on chromosome 18 affects mammary tumor latency, suggesting that it may affect mammary tumor growth. The additive interaction between *Mmom2* and *Mmom4* suggests the genes underlying these two loci do not act in the same pathway.

Humans carrying germ-line mutations on *APC* usually develop colon cancer but very few other cancer types, implying that there are tissue-specific effects of loss of APC function. Our previous data suggest that there is no association between susceptibility to mammary tumors and intestinal tumors in F1 $Apc^{Min/+}$ mice and raises a question whether there are tissue-specific modifiers of Apc^{Min} (7). Our current data show that the susceptibility to intestinal tumors in this backcross is mostly controlled by *Mom1* on chromosome 4, which has no effect on mammary tumor number or tumor latency. Conversely, the mammary modifiers show no association with intestinal tumor multiplicity. Alina et al. (31) reported that recombinant congenic strains (RCS) carrying the Apc^{Min} allele show opposite effects on intestinal and mammary tumors. These opposite effects could be due to one modifier locus with opposite effects in each tissue or the effects of tissue-specific modifiers. Our data favor the hypothesis that the observed phenotype in RCS is caused by different modifiers. The identification of tissue or tumor type–specific modifiers may aid in the identification of the genes underlying the modifier loci based on function. This also indicates the complexity of the phenotype conferred by loss of *Apc* function on different tissues.

Interaction between modifiers is frequent in colon cancer, lung cancer, and skin cancer (32–35). However, to identify low-penetrance disease-related genes presents a challenge in human and mouse genetics. The powerful genetic manipulation possible in mice makes it feasible to identify these low-penetrance genes. These low-penetrance genes might only be identified when they interact with major QTL or other low-penetrance loci to achieve a statistically significant effect. In our experiments, we used backcrosses to reduce the genetic complexity (36) because of the narrow mammary tumor number range in B6-$Apc^{Min/+}$, FVBB6 F1-$Apc^{Min/+}$, and 129X1B6 F1 $Apc^{Min/+}$ mice. Our results reveal that additive effects between modifiers not only affect mammary tumor number but also regulate mammary tumor latency. These results suggest that multiple loci control each stage of the development of mammary tumors and distinct stages are controlled by different modifier loci.

Only a few QTL affecting mammary tumor susceptibility have been mapped in mice, all of which are in the mouse mammary tumor virus (MMTV)-*PyVT* transgenic mouse model (37, 38). *Mmom1* maps near *Apmt2,* which interacts with *Apmt1* (on chromosome 15) to affect tumor latency but not tumor number (37) in *MMTV-PyVT* mice. *Mmom2* maps near *Mmtg1*, which affects total mammary tumor weight but not tumor number or tumor latency (38). However, the FVB alleles confer sensitivity in the *MMTV-PyVT* model, whereas the FVB alleles confer resistance in the $Apc^{Min/+}$ mouse model. As they affect different phenotypes with different susceptibility/resistant status, these modifier loci most likely represent different genes, although they map to a similar location. Several QTL affecting various aspects of mammary tumor development in the rat have been mapped to the orthologous regions to the loci we have mapped on chromosomes 4. 9, and 18 (39, 40). Some of the rat and mouse loci that map to orthologous regions seem to have similar effects on tumor development. However, given the imprecision of the mapping and the differences in the models, it is premature to conclude that we have mapped the same genes. However, with more precise mapping and further phenotypic analysis, it will be possible to determine whether the same genes have indeed been identified.

Mmom1 maps to the distal part of chromosome 9, which is orthologous to human 3p21-23. This region undergoes frequent loss of heterozygosity (LOH) and hypermethylation in lung cancer and breast cancer (41, 42). In addition, the *Mmom2* region is orthologous to human chromosome 9q, which has been shown to undergo LOH in about 40% of breast cancers (43). Therefore, it is possible that the loci we have identified may have a role in the development of human cancer.

B6 and FVB are two strains that are frequently used for mouse models of cancer research. As such, information about loci that alter the course of tumor development or progression can possibly be applied to other models. The identification of QTL is the first important step toward understanding the development of mammary tumors, and the characterization of modifier genes could provide valuable insights into the regulation of mammary tumors. These modifier genes could be biomarkers for early stages of breast cancer and improve the diagnosis of breast cancer.

Acknowledgments

Received 7/3/2007; revised 9/27/2007; accepted 10/8/2007.

Grant support: Department of Defense Breast Cancer Program DAMD17-02-1-0627 (A.R. Moser) and W81XWH-06-1-0413 (H. Wang).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Laura Hegge and Jared Finger for assistance with the mouse colony and backcross experiments; Dr. Norman Drinkwater for the Q-Link program and for the advice; Dr. Karl Broman for the advice on using R/qtl; Dr. Ruth Sullivan for the assistance with pathology; Meng Chen for the help with statistical analyses; and the University of Wisconsin Comprehensive Cancer Center Histology Core for assistance with processing and sectioning of tumor samples.

References

1. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.
2. Easton DF, Pooley KA, Dunning AM, et al. Genome-wide association study identifies novel breast cancer susceptibility loci. Nature 2007;447:1087–93.
3. Balmain A. Cancer as a complex genetic trait: tumor susceptibility in humans and mouse models. Cell 2002; 108:145–52.
4. Cardiff RD, Aguilar-Cordova E. Proto-neoplasia revisited: the molecular biology of mouse mammary hyperplasia. Anticancer Res 1988;8:925–33.
5. Page DL, Schuyler PA, Dupont WD, Jensen RA, Plummer WD, Jr., Simpson JF. Atypical lobular hyperplasia as a unilateral predictor of breast cancer risk: a retrospective cohort study. Lancet 2003;361: 125–9.
6. Moser A, Mattes E, Dove W, Lindstrom M, Haag J, Gould M. ApcMin, a mutation in the murine *Apc* gene, predisposes to mammary carcinomas and focal alveolar hyperplasias. PNAS 1993;90:8977–81.
7. Moser AR, Hegge LF, Cardiff RD. Genetic background affects susceptibility to mammary hyperplasias

and carcinomas in Apc^{Min}/+ mice. Cancer Res 2001; 61:3480–5.
8. Groden J, Thliveris A, Samowitz W, et al. Identification and characterization of the familial adenomatous polyposis coli gene. Cell 1991;66:589–600.
9. Nishisho I, Nakamura Y, Miyoshi Y, et al. Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. Science 1991;253:665–9.
10. Su LK, Kinzler KW, Vogelstein B, et al. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. Science 1992;256:668–70.
11. Dietrich WF, Lander ES, Smith JS, et al. Genetic identification of Mom-1, a major modifier locus affecting Min-induced intestinal neoplasia in the mouse. Cell 1993;75:631–9.
12. MacPhee M, Chepenik KP, Liddell RA, Nelson KK, Siracusa LD, Buchberg AM. The secretory phospholipase A2 gene is a candidate for the Mom1 locus, a major modifier of ApcMin-induced intestinal neoplasia. Cell 1995;81:957–66.
13. Rubinfeld B, Souza B, Albert I, et al. Association of the APC gene product with β-catenin. Science 1993;262: 1731–4.
14. Polakis P. Wnt signaling and cancer. Genes Dev 2000; 14:1837–51.
15. Michaelson JS, Leder P. β-catenin is a downstream effector of Wnt-mediated tumorigenesis in the mammary gland. Oncogene 2001;20:5093–9.
16. Schoenenberger CA, Andres AC, Groner B, van der Valk M, LeMeur M, Gerlinger P. Targeted c-myc gene expression in mammary glands of transgenic mice induces mammary tumours with constitutive milk protein gene transcription. EMBO J 1988;7:169–75.
17. Wang TC, Cardiff RD, Zukerberg L, Lees E, Arnold A, Schmidt EV. Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. Nature 1994;369: 669–71.
18. Gillett C, Smith P, Gregory W, et al. Cyclin D1 and prognosis in human breast cancer. Int J Cancer 1996;69: 92–9.
19. Furuuchi K, Tada M, Yamada H, et al. Somatic mutations of the APC gene in primary breast cancers. Am J Pathol 2000;156:1997–2005.
20. Zhang SY, Caamano J, Cooper F, Guo X, Klein-Szanto AJ. Immunohistochemistry of cyclin D1 in human breast cancer. Am J Clin Pathol 1994;102:695–8.
21. Dolled-Filhart M, McCabe A, Giltnane J, Cregger M, Camp RL, Rimm DL. Quantitative in situ analysis of β-catenin expression in breast cancer shows decreased expression is associated with poor outcome. Cancer Res 2006;66:5487–94.
22. Lin S-Y, Xia W, Wang JC, et al. β-Catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. PNAS 2000;97: 4262–6.
23. Truett GE, Heeger P, Mynatt RL, Truett AA, Walker JA, Warman ML. Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). Biotechniques 2000;29:52–4.
24. Broman KW, Wu H, Sen S, Churchill GA. R/qtl: QTL mapping in experimental crosses. Bioinformatics 2003; 19:889–90.
25. Bilger A, Bennett LM, Carabeo RA, et al. A potent modifier of liver cancer risk on distal mouse chromosome 1: linkage analysis and characterization of congenic lines. Genetics 2004;167:859–66.
26. Lan H, Kendziorski CM, Haag JD, Shepel LA, Newton MA, Gould MN. Genetic loci controlling breast cancer susceptibility in the Wistar-Kyoto rat. Genetics 2001;157: 331–9.
27. Schwarz G. Estimating the dimension of a model. Ann Stat 1978;6:461–4.
28. Li R, Lyons MA, Wittenburg H, Paigen B, Churchill GA. Combining data from multiple inbred line crosses improves the power and resolution of quantitative trait loci mapping. Genetics 2005;169:1699–709.
29. Going JJ. Stages on the way to breast cancer. J Pathol 2003;199:1–3.
30. Zhu K, Bernard LJ, Levine RS, Williams SM. Estrogen receptor status of breast cancer: a marker of different stages of tumor or different entities of the disease? Med Hypotheses 1997;49:69–75.
31. Czarnomska A, Krysiak E, Piskorowska J, et al. Opposite effects of modifiers of the ApcMin mutation in intestine and mammary gland. Cancer Res 2003;63: 4533–7.
32. Fijneman RJ, Jansen RC, van der Valk MA, Demant P. High frequency of interactions between lung cancer susceptibility genes in the mouse: mapping of Sluc5 to Sluc14. Cancer Res 1998;58:4794–8.
33. Nagase H, Mao JH, de Koning JP, Minami T, Balmain A. Epistatic interactions between skin tumor modifier loci in interspecific (spretus/musculus) backcross mice. Cancer Res 2001;61:1305–8.
34. Fijneman RJ, de Vries SS, Jansen RC, Demant P. Complex interactions of new quantitative trait loci, Sluc1, Sluc2, Sluc3, and Sluc4, that influence the susceptibility to lung cancer in the mouse. Nat Genet 1996;14:465–7.
35. Ruivenkamp CA, Csikos T, Klous AM, van Wezel T, Demant P. Five new mouse susceptibility to colon cancer loci, Scc11–15. Oncogene 2003;22:7258–60.
36. Darvasi A. Experimental strategies for the genetic dissection of complex traits in animal models. Nat Genet 1998;18:19–24.
37. Le Voyer T, Lu Z, Babb J, Lifsted T, Williams M, Hunter KW. An epistatic interaction controls the latency of a transgene-induced mammary tumor. Mamm Genome 2000;11:883–9.
38. Le Voyer T, Rouse J, Lu Z, Lifsted T, Williams M, Hunter KW. Three loci modify growth of a transgene-induced mammary tumor: suppression of proliferation associated with decreased microvessel density. Genomics 2001;74:253–61.
39. Gould KA, Tochacek M, Schaffer BS, et al. Genetic determination of susceptibility to estrogen-induced mammary cancer in the ACI rat: mapping of Emca1 and Emca2 to chromosomes 5 and 18. Genetics 2004; 168:2113–25.
40. Schaffer BS, Lachel CM, Pennington KL, et al. Genetic bases of estrogen-induced tumorigenesis in the rat: mapping of loci controlling susceptibility to mammary cancer in a Brown Norway x ACI intercross. Cancer Res 2006;66:7793–800.
41. Maitra A, Wistuba II, Washington C, et al. High-resolution chromosome 3p allelotyping of breast carcinomas and precursor lesions demonstrates frequent loss of heterozygosity and a discontinuous pattern of allele loss. Am J Pathol 2001;159:119–30.
42. Wistuba II, Behrens C, Virmani AK, et al. High resolution chromosome 3p allelotyping of human lung cancer and peneoplastic/preinvasive bronchial epithelium reveals multiple, discontinuous sites of 3p allele loss and three regions of frequent breakpoints. Cancer Res 2000;60:1949–60.
43. An H-X, Claas A, Savelyeva L, et al. Two regions of deletion in 9p23–24 in sporadic breast cancer. Cancer Res 1999;59:3941–3.

Genetic Background affects Mammary Tumor Types in *Apc*$^{Min/+}$ Mice

Hua Wang, Ruth Sullivan, Amy R Moser
Department of Human Oncology and Genetics, University of Wisconsin-Madison,

Abstract

Mammary adenocarcinomas and squamous cell carcinomas are two tumor types with distinct histological appearance. Little is known about the relationship between these two different tumor types. Here we report that different tumor types arise from the mammary glands of mice with the same inherited genetic mutation in *Apc*. B6 *Apc*$^{Min/+}$ mice develop mammary squamous cell carcinomas while FVB.B6-*Apc*$^{Min/+}$ develop mammary adenocarcinomas. Immunohistochemical (IHC) analysis revealed nuclear β-catenin accumulation in mammary alveolar hyperplasias and squamous metaplasias, suggesting a mutation in *Apc* is an early essential step for both tumor types. Nuclear β-catenin is lost during the progression from mammary alveolar hyperplasias to adenocarcinomas in FVB.B6-*Apc*$^{Min/+}$ females. Further characterization shows that membrane E-cadherin/β-catenin is differentially expressed in mammary squamous cell carcinomas and adenocarcinomas. ER-α is also lost during the progression from hyperplasias to adenocarcinomas. This observation is a first step toward understanding the genetic control of mammary tumor types.

Introduction

Canonical Wnt signaling plays an important role in animal development and tumorgenesis through its central effector β-catenin [1-3]. When Wnt signaling is absent, cytoplasmic β-catenin is phosphorylated by a complex including APC, GSK3, Axin, and Casein kinases (CK) and is degraded by the proteasome complex. When Wnt proteins bind their receptors or when mutations limit the function of the destruction complex components, β-catenin can translocate to the nucleus to bind with the LEF/TCF transcription factors , therefore regulating Wnt target genes to affect multiple biological processes including cell fate decisions, cell growth, and cell death [4].

The role of Wnt/β-catenin signaling in mammary gland development and carcinogenesis is well demonstrated in mouse models [5]. Mammary tumors from canonical Wnt/β-catenin transgenic mice have common histological characteristics, including branched ductules, an acinar component and squamous differentiation [6]. The tumor types are usually classified as either adenocarcinomas or squamous cell carcinomas. If each component in Wnt signaling exerts its effect only through the same pathway or same β-catenin/TCF complex, we would expect to observe similar mammary tumor types [7]. However mammary tumor types are quite distinct among mutants of different Wnt pathway components. All mammary tumors from ENU-treated B6 *Apc*$^{Min/+}$ mice are squamous cell carcinomas (also called pilar tumors) with keratinization, inflammatory infiltrates, and well-developed stroma [6, 8]. Mammary tumors from transgenic mice, including *Wnt1*, *Wnt10b*, *Ck2α*, and *GSK3β* as transgenes, show extensive microacinar and glandular differentiation and are generally classified as adenocarcinomas, which are

never observed in B6 *Apc*$^{Min/+}$ mice [6]. These mice are on the FVB background and transgenes are driven by MMTV promoters. Even different mutations in *β-catenin* can lead to different mammary tumor types: adenocarcinomas [9, 10] or squamous cell carcinomas [7] . There could be several explanations for these histological differences in the tumors developing in mice carrying these various mutants: a) the phenotype is gene specific or allele specific; b) chemical carcinogen treatment (ENU) induces a different type of tumor; or c) the genetic background could affect the development of mammary tumors. To begin to understand this difference, we transferred the *Apc*^{Min} allele onto the FVB background to test for the effect of genetic background on tumor development. Our hypothesis was that if genetic background controls tumor type, then we should observe adenocarcinomas in the FVB.B6-*Apc*$^{Min/+}$ mice.

Squamous cell carcinomas (SCC) represent a small percentage of all human breast carcinomas, generally with a poor prognosis. The majority of human breast carcinomas are ductal or lobular adenocarcinomas [11]. The relationship between breast SCC and adenocarcinomas is largely unknown. We have previously shown that in B6-*Apc*$^{Min/+}$ females, squamous metaplasias give rise to squamous cell carcinomas while (FVBxB6) F1 *Apc*$^{Min/+}$ females develop mixed hyperplastic and squamous metaplasias [8]. Most mammary tumors from F1 *Apc*$^{Min/+}$ mice are squamous cell carcinomas with few adenocarcinomas [8]. These observations raise the question of whether mammary alveolar hyperplasias and squamous metaplasias would develop into the same or different mammary tumor types. The hypothesis we favor is that mammary alveolar hyperplasias could progress into adenocarcinomas while squamous metaplasias could progress into the SCC, which is affected by modifiers.

Mouse models of human breast cancer help to advance our understanding of the initiation, progression, and metastasis of breast cancer and clinically relevant mouse models can be very useful for drug development. Different inbred strains have been used for mouse models and have various phenotypes even for mutations in a same gene. It is therefore important to clarify the effect of genetic background for better disease modeling. B6 and FVB are two of the mostly used inbred strains in mouse genetics and mouse models. B6 is widely used in targeted gene knockout mouse models and FVB is widely used for transgenic analysis [12, 13]. The clarification of how B6 and FVB backgrounds affect mammary tumor development can help us to build a better mouse model.

Materials and Methods

Animals

All mice were maintained in the Animal Care Facility of University of Wisconsin Medical School. All procedures were approved by the ACUC. Female C57BL/6J (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and FVB/NT (FVB) mice are from Taconic Labs (Hudson, NY). The C57BL/6J *Apc*$^{Min/+}$ (B6 *Apc*$^{Min/+}$) mice were maintained by crossing wild type B6 females from The Jackson Laboratory with B6 *Apc*$^{Min/+}$ males.

For production of FVB.B6-*Apc*$^{Min/+}$ congenic mice, *Apc*$^{Min/+}$ males were backcrossed to FVB wild type females and *Apc*$^{Min/+}$ males were selected in each generation. N5 FVB.B6-*Apc*$^{Min/+}$ females were used in this study. N5 FVB- females were treated with ENU as described for B6- *Apc*$^{Min/+}$ mice [8]. All females were palpated every week and sacrificed when they became sick or after 300 days.

Histology and Immunohistochemistry

Mammary glands and tumors were collected at the time of euthanasia and fixed in 10% buffered formalin overnight and then transferred to 70% ethanol for storage. Mammary glands were stained with carmine as described before [8] and alveolar hyperplasias were excised from fixed and stained mammary glands. Both mammary alveolar hyperplasias and mammary tumors are then embedded and sectioned for histological evaluation.

The primary antibodies used in this study were β-catenin (1:50, BD Transduction Labs), E-cadherin (1:500, clone 36, BD Transduction Labs) and Estrogen receptor-α (1:300, Santa Cruz, MC-20).

Immunohistochemical analysis was performed as described in the manufacturer's instructions (Catalog NO. PK2202, Vector Laboratories, Burlingame, CA). In brief, 5-μm paraffin-embedded sections were deparaffinized in xylene, and then rehydrated through graded alcohols into distilled water. Antigen retrieval was performed by microwaving the slides in Sodium Citrate buffer (pH 6.0) for 20 min. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in PBS for 10 minutes at room temperature, and the slides were blocked with IG blocking buffer for 1 h at room temperature. Sections were incubated with primary antibodies for 45 minutes at room temperature, followed by a biotinylated secondary antibody at a 1:300 dilution for 1 hour at room temperature. Antigen-antibody complexes were detected with the avidin-biotin peroxidase method using 3,3'-diaminobenzidine (DAB) as a chromogenic substrate. Immunostained sections were slightly counterstained with hematoxylin or methyl green and then examined by light microscopy.

Statistical Analysis

All analysis was carried out using the MSTAT program provided by Dr. Norman Drinkwater at University of Wisconsin-Madison. Kaplan-Meier analysis was performed for the tumor latency and the Log-rank test was used for pair-wise comparison of tumor latency.

Results

FVB.B6-*Apc*$^{Min/+}$ Females develop mammary Adenocarcinomas

We previously identified several dominant modifiers from FVB which confer resistance to mammary tumor number or tumor latency. To evaluate the joint effect of all modifiers,

the *Apc*^{Min} allele was transferred onto the FVB genetic background and N5 FVB.B6-*Apc*$^{Min/+}$ females were treated with ENU. No difference was observed between (FVBxB6) F1-*Min/+* and N5 FVB.B6-*Apc*$^{Min/+}$ females with respect to mammary hyperplasia number (3.4 ± 2.1 Vs 3.6 ± 1.8, P>0.05), and total tumor number per mouse (1 ± 0.9 Vs 1 ± 1.0, P>0.05). However N5 congenic FVB.B6-*Apc*$^{Min/+}$ females had a significantly longer tumor latency than F1-*Apc*$^{Min/+}$ females (Figure 3-1, P<0.01)

The most interesting phenotype we observed was that the majority of mammary tumors from the N5 FVB.B6-*Apc*$^{Min/+}$ females were adenocarcinomas (Figure 3-2A and 3-2E), in contrast to 100% Squamous cell carcinomas in B6-*Apc*$^{Min/+}$ females (Figure 3-2B and 3-2D). Among a total of 23 mammary tumors analyzed, 14/23 were classified as adenocarcinomas (Figure 3-2E) and 9/23 were classified as adenosquamous carcinomas (Figure 3-2G) which show a mixed phenotype. We also examined a total of 17 mammary alveolar hyperplasias from 17 different FVB.B6-*Apc*$^{Min/+}$ females. All mammary alveolar hyperplasias showed focal avelolar structures with isolated squamous differentiation (Figure 3-2E).

Deregulation on β-catenin in Mammary Tumor Types

We hypothesized that ENU treatment would induce mutations in the wild-type allele of *Apc* and activate the canonic Wnt signaling pathway to initiate mammary lesions. As a surrogate for testing for loss of the wild-type allele of *Apc*, we tested for the localization of β-catenin. In both squamous metaplasias from B6-*Apc*$^{Min/+}$ mice and focal alveolar hyperplasias from FVB.B6-*Apc*$^{Min/+}$ mice, β-catenin was found to accumulate in the nucleus (Figure 3-3A and 3-3C). All alveolar hyperplasias retained membrane β-catenin staining (Figure 3-3A), which was lost in the squamous metaplasias (Figure 3-3C).

Mammary SCC and adenocarcinomas also showed different β-catenin expression patterns. In the SCC from B6-*Apc*$^{Min/+}$ females, we observed strong β-catenin staining in the nucleus, similar to β-catenin staining in the squamous metaplasias (Figure 3-3D). In the majority of adenocarcinomas or in the adenocarcinomatous parts of adenosquamous tumors (15/16) from FVB-*Apc*$^{Min/+}$ mice, β-catenin shows strong membrane staining (Figure 3-3B and 3-3F), but little to no nuclear accumulation of β-catenin (Figure 3-3B and 3-3F). In only one adenocarcinoma (1/16) was nuclear β-catenin staining observed (Figure 3-3E). In the squamous parts of the adenosquamous tumors from FVB.B6-*Apc*$^{Min/+}$ mice, β-catenin was detected in the nucleus, similar to the β-catenin staining in the SCC (Figure 3-3F).

In addition to its role in the canonical Wnt pathway, β-catenin can bind with E-cadherin to regulate cell adhesion, which affects many cellular process including cell growth, cell differentiation and cell migration. [14]. In the light of physical interaction between β-catenin and E-cadherin, we did immunohistochemical staining on the mammary SCC and adenocarcinomas for the presence of E-cadherin. E-cadherin showed a similar membrane staining pattern as β-catenin in the SCC and the adenocarcinomas. In the squamous metaplasias and the SCC, membrane E-cadherin staining is lost (Figure 3-4C and 3-4D)

while E-cadherin membrane staining remains strong in the mammary alveolar hyperplasias and the adenocarcinomas (Figure 3-4A and 3-4B).

To characterize the tumors with respect to ERα status, we stained the mammary lesions with ER-α antibody. The squamous metaplasias and the SCC from B6.*Apc*$^{Min/+}$ mice were ER-negative (Figure 3-4H and 3-4I). The mammary alveolar hyperplasias from FVB.B6-*Apc*$^{Min/+}$ mice were ER-positive (Figure 3-4E) while most mammary adenocarcinomas (17/18) were ER-negative (Figure 3-4F). The only adenocarcinoma with nuclear ER staining was also positive for β-catenin (Figure 3-4G).

Discussion

In conclusion, we provide strong evidence that genetic background can affect mammary tumor types: FVB.B6-*Apc*$^{Min/+}$ females developed mammary adenocarcinomas or adenosquamous carcinomas with a long tumor latency while B6 *Apc*$^{Min/+}$ females develop mammary squamous cell carcinomas with a short tumor latency. The (FVBB6) F1 *Apc*$^{Min/+}$ mice mostly develop SCC with an intermediate tumor latency. We previously mapped tumor latency modifiers in an (FVBxB6) x B6 backcross and the data here suggest that the resistant alleles of these modifiers act in a semi-dominant way. The similar susceptibility to mammary alveolar hyperplasias and tumor number between FVB *Apc*$^{Min/+}$ and (FVBxB6) F1 *Apc*$^{Min/+}$ suggests that the resistant alleles of the modifiers affecting tumor number are likely fully dominant. These data also support the hypothesis that the modifiers affect mammary tumor development after the stage of ENU treatment (initiation). In the backcross (FVBxB6) F1 to B6-*Apc*$^{Min/+}$, we examined the histology of more than 400 mammary tumors but did not observe any mammary adenocarcinomas (Data not shown). This suggests that the modifier alleles from FVB act recessively to suppress SCC.

The mammary tumor type in FVB.B6-*Apc*$^{Min/+}$ mice is the same as in *Wnt1* and *β–catenin* transgenic mice on the FVB background [6]. This observation strongly supports the hypothesis that the genetic background can affect mammary tumor types in *Apc*$^{Min/+}$ mice and suggests the genetic background may have a similar effect on tumor development in other Wnt components transgenic mice. If this were true, it would partially explain the different mammary tumor types observed in the different Wnt-components transgenic mice.

Our results show that ENU treatment can induce mutations in *Apc* as evident through the nuclear accumulation of β-catenin in both mammary alveolar hyperplasias and metaplasias. This result is consistent with previous reports that ecotopic activation of *β-catenin* can cause overproliferation of epithelial cells and transdifferentiation of mammary epithelial cells [15-17]. These results are against several possibility to explain the different mammary tumor types we observed. Because ENU treatment can induce mutations in *Apc* and does not target different cell types, we would expect both ductal epithelial cells and alveolar epithelial cells to have the same chance of having mutations in *Apc*. Other Wnt components transgenes were usually driven by the MMTV promoter so the difference in mammary tumor types could be due to the selection of hormone-

responsive cells. The data here do not support this hypothesis. The histological difference is also not ENU dependent because both FVB.B6-*Apc*$^{Min/+}$ and B6-*Apc*$^{Min/+}$ were treated with ENU. Our data also exclude the possibility that the distinct mammary tumor types in the FVB and B6 *Apc*^{Min}/+ mice are due to different APC expression level because both mammary tumor types lack functional APC. Also, the similar mammary tumor types observed in *Apc*$^{Min/+}$, MMTV-*Wnt1*, MMTV-*Wnt10b* MMTV-*GSK3 β*, and MMTV-*β-catenin* females on the FVB background suggest the phenotype is not gene or allele specific [6].

It is particularly interesting to note that different mammary tumor types can arise from different mutations in β-catenin. An N-terminal truncated β-catenin which lacks amino acids 1-89 (delta-N89) [10] or lacks amino acids 1-90 (delta-N90) [9] under the control of MMTV-LTR resulted in the mammary alveolar hyperplasias and adenocarcinomas in virgin or parous females. These mice were generated by pronuclear injection and were maintained on the FVB background. However, female mice carrying a floxed *β-catenin* under WAP-*Cre* or MMTV-*Cre* mediated recombination developed mammary squamous metaplasias and SCC. In these mice, the floxed allele has LoxP sequence flanking exon 3 which encodes amino acids 5-80 so these different *β-catenin* alleles result in proteins with similar deletions. Because the floxed *β-catenin* mutant was originally maintained on the C57BL/6N background [18], this raises the possibility that the observed SCC in the floxed β-catenin mutant are due to the genetic background difference. However, this hypothesis cannot be confirmed as the authors did not state clearly the genetic background of the mice used in their study [17]. To get a clear answer to these questions, it would be necessary to backcross the mutants to the same genetic background to compare the phenotypes

We noticed different β-catenin expression pattern between adenocarcinomas and SCC. In both the squamous metaplasias and the mammary alveolar hyperplasias, nuclear staining of β-catenin is clear. However, nuclear β-catenin is more uniform and appears more intense in the squamous metaplasias and the SCC than in the mammary alveolar hyperplasias. So the level of nuclear β-catenin could be important in the cell fate decisions [19]. Our data also show that the membrane E-cadherin/ β-catenin complex is lost in SCC while remains intact in alveolar hyperplasias and adenocarcinomas. The loss of the E-cadherin/ β-catenin membrane complex could contribute to increased nuclear β-catenin [14]. But it remains unclear whether the loss of the E-cadherin/ β-catenin complex causes the squamous transdifferentiation or is a result of squamous transdifferentiation. Lack of E-cadherin is frequently observed in human squamous cell carcinomas of the skin, esophagus, head and neck [20-22], suggesting that lack of E-cadherin is a common event in squamous cell carcinomas. These data suggest that membrane E-cadherin/β-catenin complex is involved genetic control of SCC and adenocarcinomas.

Several lines of evidence support the hypothesis that mammary alveolar hyperplasias would progress into adenocarcinomas and squamous metaplasias would progress into SCC depending on the genetic background. First is the similar histological appearance. Squamous metaplasias and SCC are both well differentiated and contains lots of keratins.

Alveolar hyperplasias and adenocarcinomas remain contain acinar structures in our study. Second is that the lesion types are strain-dependent. B6 *Apc*$^{Min/+}$ females only develop squamous metaplasias and SCC while FVB.B6-*Apc*$^{Min/+}$ preferentially develop mammary alveolar hyperplasias and adenocarcinomas. Thirdly, preneolastic lesions and tumors share the similar E-cadherin/ β-catenin expression. Memebrane E-cadherin/ β-catenin expression remains well in hyperlasias and adenocarcinomas while the E-cadherin/ β-catenin expression is lost in the squamous metaplasias and SCC.

The observation that nuclear β-catenin and ER-α are lost during the progression from mammary alveolar hyperplasias to mammary adenocarcinomas is surprising and interesting. The mammary adenocarcinomas from MMTV-*β-catenin* and MMTV-*GSK3 β* are also ER-negative[23, 24]. This result is consistent with the observation in human breast squamous cell carcinomas[11]. This indicates that additional genetic change is required for the progression from alveolar hyperplasias and tumors. The adenocarcinomas that develop in MMTV-*Wnt1* and MMTV-ΔNβ-catenin mice usually require a long tumor latency [9, 10, 25], which indicates the need for further genetic events. Chodosh and colleagues reported that the *P53* and *Ras* signaling pathways can affect mammary adenocarcinomas development in *Wnt1* transgenic mice [26, 27]. Changes in the expression level of Neu/ErbB2 or P53 can affect ER status in *Wnt1* transgenic mice [24]. It will be intriguing to investigate what additional genetic changes exist between SCC and adenocarcinomas in *Apc*$^{Min/+}$ mice.

So what are the genetic regulators that affect mammary tumor types in *Apc*$^{Min/+}$ mice? The question still remains largely unknown. Our data shows that the same focal alveolar hyperplasias with isolated squamous differentiation were observed in (FVBxB6) F1 *Apc*$^{Min/+}$ and FVB.B6-*Apc*$^{Min/+}$ females while most tumors are SCC in (FVBxB6) F1 *Apc*$^{Min/+}$ females and most tumors are adenocarcinomas in (FVBxB6) F1 *Apc*$^{Min/+}$ females. The favored explanation is that the progression from hyperlaisas to adenocarcinomas is suppressed in (FVBxB6) F1 *Apc*$^{Min/+}$ females while the progression from squamous metaplasias to tumors is suppressed in FVB.B6-*Apc*$^{Min/+}$ females. The Schuler lab has shown that overexpression of *prolactin* (*PRL*) can change the mammary tumor types from SCC to adenocarcinomas in (FVBxB6) F1 *Apc*$^{Min/+}$ mice (L. Schuler and K. O'Leary, personal communication). So the prolactin pathway could modify mammary tumor types. Other pathways involved in this histological determination remain to be explored.

Overall, our results support that genetic background can affect mammary tumor types in *Apc*$^{Min/+}$ mice. Our data also suggest that SCC and adenocarcinomas develop from the different preneoloastic lesions. This information would be the first step toward understanding the genetic control of mammary tumor types.

Figure 1 The Effect of Genetic Background on Mammary Tumor Latency

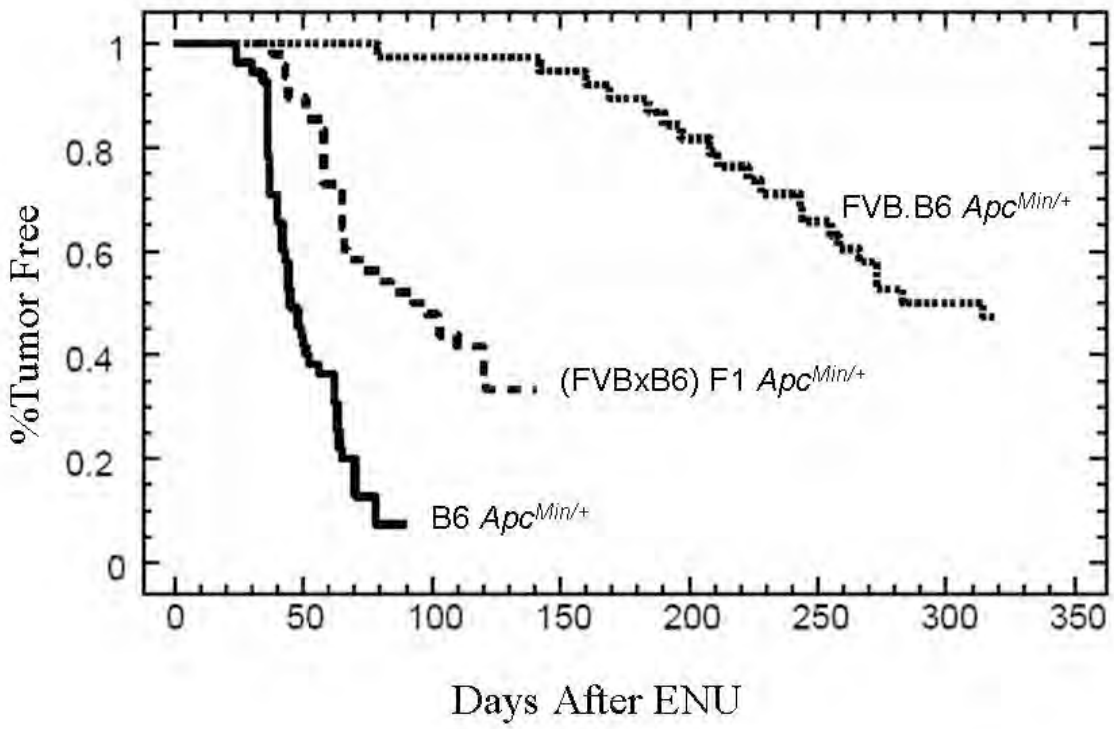


Figure 2: Histological analysis of mammary lesions from *Apc*$^{Min/+}$ mice with H&E staining.
2A) Mammary tumors from FVB.B6-*Apc*$^{Min/+}$ mice; 2B) Mammary tumors from B6*Apc*$^{Min/+}$ mice; 3-2C) Mammary squamous metaplasias from B6 *Apc*$^{Min/+}$ mice; 2D) Mammary squamous cell carcinomas from B6-*Apc*$^{Min/+}$ mice; 2E) Mammary hyperplasias from FVB.B6-*Apc*$^{Min/+}$ mice; 2F) Mammary adenocarcinomas from FVB.B6-*Apc*$^{Min/+}$ mice; 2G) Mammary adenosquamous carcinomas from FVB.B6-*Apc*$^{Min/+}$ mice.

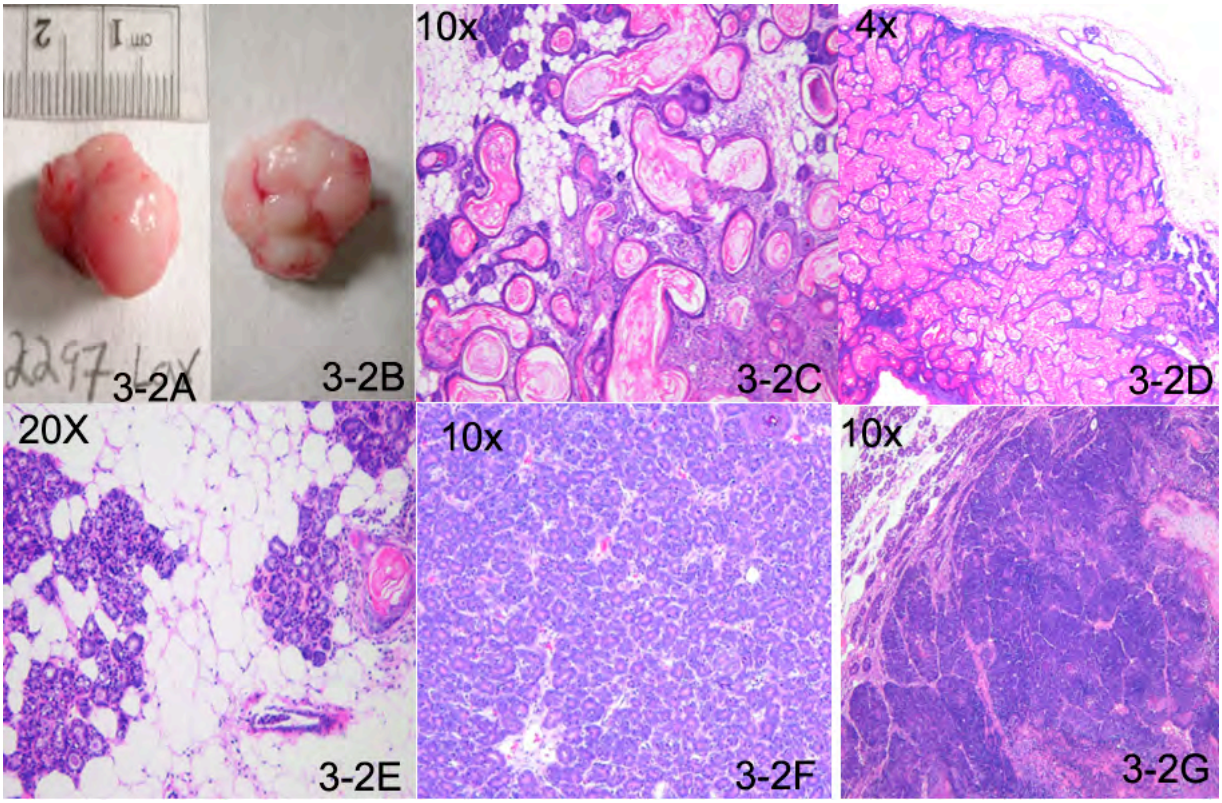


Figure 3: β-catenin staining in mammary lesions from *Apc*$^{Min/+}$ mice. 3A) Membrane and nuclear β-catenin staining in mammary hyperplasias compared with normal mammary gland from FVB.B6-*Apc*$^{Min/+}$ mice; 3B) Membrane β-catenin staining in mammary adenocarcinomas from FVB.B6-*Apc*$^{Min/+}$ mice; 3C) Nuclear β-catenin staining in mammary squamous metaplasias compared with normal mammary gland from B6-*Apc*$^{Min/+}$; 3D) β-catenin staining in mammary squamous cell carcinomas from B6-*Apc*$^{Min/+}$. In the hair shaft structure, β-catenin accumulates in both cytoplasm and nuclear. In the less transdifferentiated cells, β-catenin is mostly in nucleus. In the well-transdifferentiated cells, no β-catenin is detected. 3E) only one mammary adenocarcinoma (1/16) from FVB.B6-*Apc*$^{Min/+}$ mice show both membrane and nuclear β-catenin staining; 3F) β-catenin staining in adenosquamous carcinomas: adenocarcinoma parts and squamous parts.

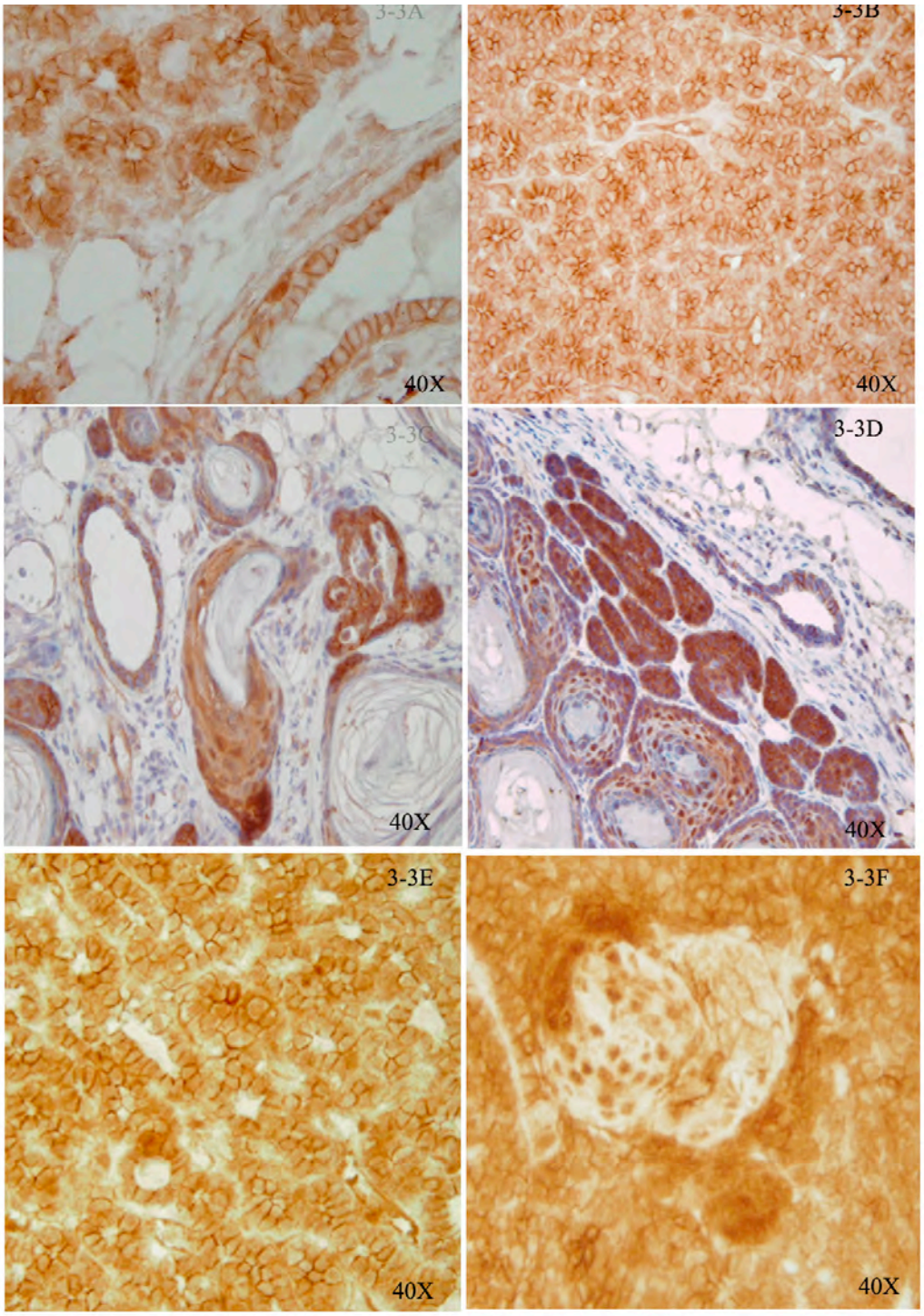
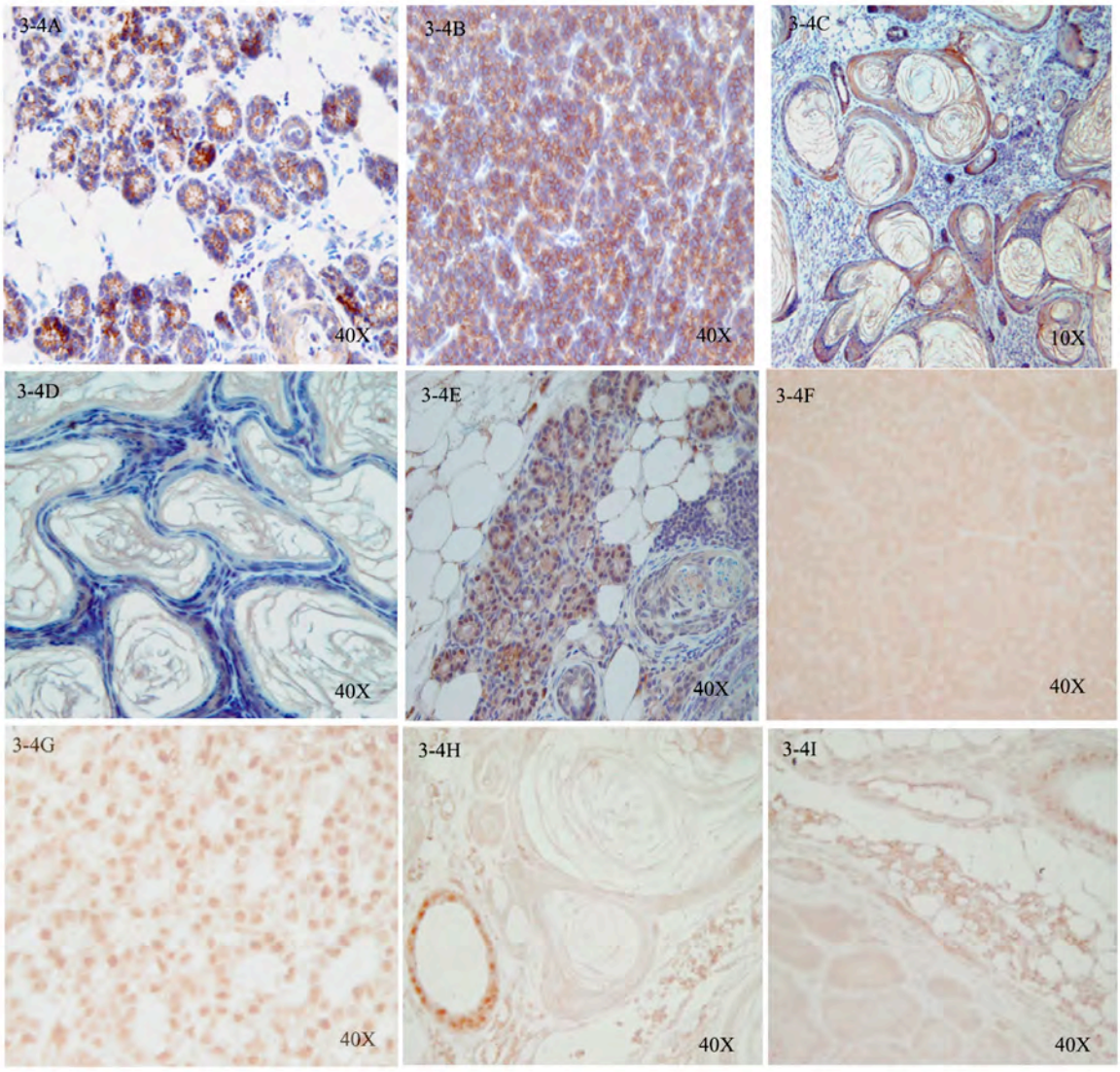

3-3A
40X
3-3B
40X
3-3C
40X
3-3D
40X
3-3E
40X
3-3F
40X

Figure 4: E-cadherin and ER-α staining in mammary lesions from *Apc*$^{Min/+}$ mice. Mammary hyperplasias (4A) and adenocarcinomas (4B) show strong membrane E-cadherin staining. Mammary squamous metaplasias (4C) and squamous cell carcinomas (4D) lost E-cadherin staining. Mammary hyperplasis is ER-α positive (4E) and most (17/18) mammary adenocarcinomas are ER-α negative (4F). Only one mammary adenocarcinoma is ER-α positive (4G). Both squamous metaplasia (4H) and squamous cell carcinomas (4I) are ER-α negative.



Reference

1. Lustig, B. and J. Behrens, *The Wnt signaling pathway and its role in tumor development.* J Cancer Res Clin Oncol, 2003. **129**(4): p. 199-221.
2. Cadigan, K.M. and R. Nusse, *Wnt signaling: a common theme in animal development.* Genes Dev., 1997. **11**(24): p. 3286-3305.
3. van Amerongen, R. and A. Berns, *Knockout mouse models to study Wnt signal transduction.* Trends Genet, 2006. **22**(12): p. 678-89.
4. Logan, C.Y. and R. Nusse, *The Wnt signaling pathway in development and disease.* Annu Rev Cell Dev Biol, 2004. **20**: p. 781-810.
5. Turashvili, G., et al., *Wnt signaling pathway in mammary gland development and carcinogenesis.* Pathobiology, 2006. **73**(5): p. 213-23.
6. Rosner, A., et al., *Pathway pathology: histological differences between ErbB/Ras and Wnt pathway transgenic mammary tumors.* Am J Pathol, 2002. **161**(3): p. 1087-97.
7. Miyoshi, K., et al., *Activation of different Wnt/beta-catenin signaling components in mammary epithelium induces transdifferentiation and the formation of pilar tumors.* Oncogene, 2002. **21**(36): p. 5548-56.
8. Moser, A.R., L.F. Hegge, and R.D. Cardiff, *Genetic background affects susceptibility to mammary hyperplasias and carcinomas in Apc(min)/+ mice.* Cancer Res, 2001. **61**(8): p. 3480-5.
9. Michaelson, J.S. and P. Leder, *beta-catenin is a downstream effector of Wnt-mediated tumorigenesis in the mammary gland.* Oncogene, 2001. **20**(37): p. 5093-9.
10. Imbert, A., et al., *Delta N89 beta-catenin induces precocious development, differentiation, and neoplasia in mammary gland.* J Cell Biol, 2001. **153**(3): p. 555-68.
11. Hennessy, B.T., et al., *Squamous cell carcinoma of the breast.* J Clin Oncol, 2005. **23**(31): p. 7827-35.
12. Taketo, M., et al., *FVB/N: An Inbred Mouse Strain Preferable for Transgenic Analyses.* Proceedings of the National Academy of Sciences, 1991. **88**(6): p. 2065-2069.
13. Seong, E., et al., *To knockout in 129 or in C57BL/6: that is the question.* Trends Genet, 2004. **20**(2): p. 59-62.
14. Nelson, W.J. and R. Nusse, *Convergence of Wnt, beta-catenin, and cadherin pathways.* Science, 2004. **303**(5663): p. 1483-7.
15. Miyoshi, K. and L. Hennighausen, *beta-catenin: a transforming actor on many stages.* Breast Cancer Research, 2003. **5**(2): p. 63-68.
16. Gat, U., et al., *De novo hair follicle morphogenesis and hair tumors in mice expressing a truncated beta-catenin in skin.* Cell, 1998. **95**(5): p. 605-614.
17. Miyoshi, K., et al., *Activation of beta-catenin signaling in differentiated mammary secretory cells induces transdifferentiation into epidermis and*

squamous metaplasias. Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(1): p. 219-224.
18. Harada, N., et al., *Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene.* Embo J, 1999. **18**(21): p. 5931-42.
19. Gallagher, R.C., et al., *Inactivation of Apc perturbs mammary development, but only directly results in acanthoma in the context of Tcf-1 deficiency.* Oncogene, 2002. **21**(42): p. 6446-57.
20. Brouxhon, S., et al., *Sequential down-regulation of E-cadherin with squamous cell carcinoma progression: Loss of E-cadherin via a prostaglandin E-2-EP2-dependent posttranslational mechanism.* Cancer Research, 2007. **67**(16): p. 7654-7664.
21. Lioni, M., et al., *Dysregulation of claudin-7 leads to loss of E-cadherin expression and the increased invasion of esophageal squamous cell carcinoma cells.* American Journal of Pathology, 2007. **170**(2): p. 709-721.
22. Schipper, J.H., et al., *E-Cadherin Expression in Squamous-Cell Carcinomas of Head and Neck - Inverse Correlation with Tumor Dedifferentiation and Lymph-Node Metastasis.* Cancer Research, 1991. **51**(23): p. 6328-6337.
23. Farago, M., et al., *Kinase-inactive glycogen synthase kinase 3 beta promotes Wnt signaling and mammary tumorigenesis.* Cancer Research, 2005. **65**(13): p. 5792-5801.
24. Zhang, X.M., et al., *Estrogen receptor positivity in mammary tumors of Wnt-1 transgenic mice is influenced by collaborating oncogenic mutations.* Oncogene, 2005. **24**(26): p. 4220-4231.
25. Tsukamoto, A.S., et al., *Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice.* Cell, 1988. **55**(4): p. 619-25.
26. Gunther, E.J., et al., *Impact of p53 loss on reversal and recurrence of conditional Wnt-induced tumorigenesis.* Genes & Development, 2003. **17**(4): p. 488-501.
27. Jang, J.W., R.B. Boxer, and L.A. Chodosh, *Isoform-specific ras activation and oncogene dependence during MYC- and Wnt-induced mammary tumorigenesis.* Molecular and Cellular Biology, 2006. **26**(21): p. 8109-8121.